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Predicting Dose-Response Relationships of Acute Cadmium Hepatoxicity and Metallothionein Regulation in the Rat Via In Vitro to In Vivo Extrapolation

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14. ABSTRACT
The purpose of this effort was to utilize cadmium, a known toxicant of environmental and occupational concern, to develop an understanding of the relationships between chemical kinetics (rates of chemical movement into the body) and cellular dynamics (cellular response to chemical entering the cells) in order to predict early target organ toxicity and refine/validate a biologically-based kinetic model. This effort involved three studies. The first measured cadmium kinetics over 24 hours in male Fischer 344 rats dosed intravenously with 0.0, 0.5, 1.0, 2.0 or 3.0 mg/kg Cd. Serum liver enzymes and histology were also monitored to determine the extent of hepatic damage. The second evaluated metallothionein (MT) mRNA regulation in these rats in response to the Cd toxicity. MT isoforms I and II were found to be separately regulated. In the third, male F344 rat hepatocytes were isolated and exposed to Cd *in vitro* at doses of 0 µM, 5 µM, 10 µM and 15 µM. MT mRNA expression in these hepatocytes was not similar to the *in vivo* response.

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PREFACE

This research was accomplished at the Applied Biotechnology Branch, Human Effectiveness Directorate of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under John J. Schlager, Branch Chief. The contractors were under the direction of Darol Dodd (Alion Science and Technology, Inc. (formerly ManTech Environmental Technology, Inc.), Dayton, OH, Contract # F-33615-00-C-6060) and Peter Lurker (Operational Technologies Corp., Dayton, OH, Contract # F33601-02-F-A211). Funding was provided by the Air Force Office of Scientific Research (AFRL/AFOSR) under the program management of Walter Kozumbo, Ph.D. This technical report was written as a final product for AFRL Workunit 2312A206.

A portion of this work was performed to fulfill obligations toward a Masters Degree in Pharmacology and Toxicology at Wright State University, Dayton, OH. All thesis work was performed in FY03; the thesis was defended in Sep. 2003. The thesis, "The two isoforms of rat metallothionein are coordinately regulated *in vivo*", by Diane M. Todd is available at Wright State University only. Additionally, the thesis conclusions were presented as a poster at the 2004 Society of Toxicology annual meeting in Baltimore, MD, under the title "The two isoforms of rat metallothionein are not coordinately regulated *in vivo*" and authored by D. Todd, V. Chan, J. Gearhart, D. Mahle, N. DelRaso, and J.M. Frazier (*Toxicol. Sci.* 78 (S-1):60). The poster was also presented at the 2004 Toxicology and Risk Assessment Conference in West Chester, OH.

All animals used in this study were handled in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1996, as amended.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREDICTING DOSE-RESPONSE RELATIONSHIPS OF ACUTE CADMIUM HEPATOXICITY AND METALLOTHIONEIN REGULATION IN THE RAT VIA *IN VITRO* TO *IN VIVO* EXTRAPOLATION

INTRODUCTION

A major objective of toxicology is to predict the *in vivo* toxicological consequences of human exposure to pure chemicals, complex mixtures and commercial formulations. Historically, the experimental approach to this goal has been to investigate toxicological processes in whole animal models and extrapolate the results obtained to predict human risk using various extrapolation procedures (high dose - low dose extrapolation, interspecies extrapolation and route-to-route extrapolation) and default assumptions.

In recent years *in vitro* methods have received greater attention with respect to their possible application in chemical safety evaluation and risk assessment (Jolles and Cordier, 1992). One major limitation to the broader application of *in vitro* toxicity testing methods is the lack of scientifically validated techniques for the extrapolation of *in vitro* derived data to the *in vivo* situation. Successful approaches to this issue must be based on a solid foundation in mechanistic toxicology. Any proposed *in vitro* - *in vivo* extrapolation procedure can only be rigorously validated in animal models where appropriate experimental studies can be conducted. Ultimately, the validated extrapolation procedures are to be applied to quantitatively predicting target organ toxicity in human populations based on *in vitro* toxicity testing in human cells.

In order to predict the time course of the toxic response of an organism following exposure to a chemical, it is necessary to define the time-dependent exposure-response relationship for that particular chemical. The exposure-response relationship is a quantitative expression describing the expected responses of an organism given a particular level of exposure to a test chemical. Various physical, chemical and biological processes interact to connect the exposure of the organism to the ultimate expression of adverse effects (Frazier, 1992). The process starts with exposure to the material and subsequent absorption into the biological system. The various processes that determine the delivered dose of the active form of the toxicant to the target tissue - absorption, distribution, metabolism, storage and excretion - are collectively referred to as kinetics.

Cellular dynamics is a generalized concept describing the response to the chemical at the cellular level. It begins with the interaction of the active form of the toxicant with its molecular target. The physical-chemical interaction of the active form of the toxicant with the molecular target, whether reversible as in the case of competitive enzyme inhibition or irreversible as in the case of protein or DNA adducts, is the driving force which generates the biological response to the toxicant. The alteration of the molecular target sets off a cascade of events that propagates at the molecular level to characterize the response at the cellular level. Repair and turnover of altered molecular targets will have a significant impact on the magnitude of the resulting cellular damage. Altered structure and function at the cellular level progressively impacts on higher and higher levels of biological organization.

Cadmium

Cadmium was selected as the model toxicant for this study for several reasons. First, cadmium (Cd) ranks close to lead (Pb), arsenic (As) and mercury (Hg) in toxicological importance due to increasing levels in the environment (Friberg *et al.*, 1986). A major source of Cd ingestion in animals and humans has been due to food contamination, namely from grains and shellfish. Also, industrial activity can significantly increase the distribution of Cd in the environment. Occupational exposure usually occurs from dust and aerosols that are typically produced during the smelting and refining of metal ores, electroplating or welding and during the manufacture of pigments, plastic stabilizers, and nickel-cadmium batteries, causing pulmonary carcinogenesis (IARC, 1993). Cigarette smoke and contaminated food, water and air represent other possible sources of Cd exposure (Elinder, 1986).

Second, the toxicokinetics of cadmium have been extensively investigated, which provides a strong basis from which to understand quantitatively the mechanisms of Cd toxicity. The kinetic database required to develop a biologically-based kinetic (BBK) model for cadmium is sufficient for initial conceptual development of the model as well as providing overall constraints on many of the kinetic parameters. Although the existing kinetic database is large, specific *in vivo* experimental studies are needed to fine tune and validate a BBK model.

Third, the hepatotoxicity of cadmium is well-documented (Dudley *et al.*, 1982; Theocharis *et al.*, 1991; Klaassen and Liu, 1997). Cd is classified as carcinogenic to humans and animals (IARC, 1993). Acute exposure to Cd has been associated with damage to the testes, lungs and liver, while chronic exposure can lead to emphysema, obstructive airway disease, permanent renal dysfunction, bone disorders, and immunotoxicity (Friberg *et al.*, 1986). After parenteral administration, the liver accumulates 60% of administered Cd (Frazier and Puglese, 1978; Cain and Skilleter, 1980); the liver is the main target of Cd toxicity (Meek, 1959; Dudley *et al.*, 1982). A hepatotoxic dose of Cd will cause parenchymal cell swelling and cytoplasm eosinophilia within 1 hour after injection, while severe necrosis is evident 10-20 hours later (Dudley *et al.*, 1982, 1984), and death due to liver failure can occur within 24 hours (Hoffmann *et al.*, 1975; Dudley *et al.*, 1982, 1984; Goering and Klassen, 1983). The wealth of knowledge regarding Cd toxicity will facilitate preliminary comparisons of *in vitro* responses with *in vivo* data.

Fourth, the *in vitro* toxicity of cadmium in hepatocyte cultures has been extensively investigated, forming the basis of the *in vitro* studies described here (Stacey *et al.*, 1980; Santone *et al.*, 1982). Fifth, cadmium salts are highly soluble, exist in one oxidation-reduction state and are non-volatile, thus eliminating the confounding issues related to insoluble chemicals, inorganic chemicals that have multiple oxidation-reduction states or volatile toxicants. Based on these reasons, cadmium was selected as the model toxicant for this study.

Although the toxicity of cadmium is well established in the literature, the mechanism of cadmium cytotoxicity still remains to be elucidated. Based on the established literature, it is apparent that factors that affect the rate of uptake and intracellular distribution of cadmium in cells will have an impact on the degree of cytotoxicity induced. Although the level of free cadmium in hepatocytes has been suggested to be the determining factor in predicting toxicity (DelRaso *et al.*, 2003), this hypothesis has not been conclusively tested since it is extremely difficult to measure the free intracellular concentration. The concentration of non-metallothionein bound cadmium has been used as an indirect measure of "reactive cadmium" in the cell (Din and Frazier, 1985).

A confounding factor in trying to understand the quantitative relationships between intracellular cadmium kinetics and cytotoxicity is the lack of consistency in experimental designs used to

investigate the kinetics of cadmium uptake and accumulation. A survey of the literature reveals that a variety of rat strains, culture systems, culture/exposure media and exposure durations have been utilized to investigate cadmium kinetics in hepatocytes. This makes it extremely difficult to interpret study results and extract quantitative parameters. Very few studies, if any, have attempted to determine how culture conditions affect intracellular free cadmium concentrations and thereby modulate toxicity. In addition, few attempts have been made to use *in vitro* data to estimate the free concentration of cadmium delivered to the liver *in vivo* as a result of a particular cadmium exposure and how it relates to toxicity.

Metallothionein

Exposure of biological organisms to toxic chemicals in the environment has been a common occurrence over time. Due to these exposures, organisms from bacteria to humans have developed defense mechanisms to handle toxic elements such as ionic Cd. One category of defense mechanism is the induction of unique detoxifying proteins and enzymes. Metallothionein (MT) is one such inducible protein that is involved in the non-covalent chelation and detoxification of heavy metals in general and cadmium in particular.

MTs are found in nearly all-living organisms and in most tissues. The liver, kidney, intestine and pancreas have been shown to contain the highest concentrations of mammalian MT in the body (Cousins, 1985; Bremner and Beattie, 1990; Nath *et al.*, 1988; Hamer, 1986). MT is also present in the brain. MT is primarily found in the cytosol of cells but immunohistochemical studies have shown increased MT expression in both the cytoplasm and nucleus of rapidly proliferating cells (Miles *et al.*, 2000; Davis and Cousins, 2000). The exact function of nuclear MT is unknown but it has been postulated that it may protect DNA from oxidative damage or regulate the supply of zinc (Zn) to crucial enzymes and transcription factors necessary for cell division (Moffatt and Denizeau, 1997; Cherian and Apostolova, 2000; Ogra and Suzuki, 2000).

MT has many functions including detoxification of toxic metals (Cd and Hg), homeostasis of essential metals (Zn and copper (Cu)), and protection against oxidative damage caused by free radicals. Metals bind to MT as metal-thiolate (mercaptid) complexes and form regional clusters within the tertiary protein structure (Hamer, 1986). Even though as many as 18 different metal ions may associate with MT, the metal content of endogenous MTs consists of primarily Zn, Cu or Cd. In Cd toxicity, MT plays a protecting role, not just against hepatotoxicity, but also nephrotoxicity, hematotoxicity, immunotoxicity and bone damage (Klassen and Choudhuri, 2000). MT plays an important role in Cd detoxification (Durnam and Palmiter, 1987) while protecting against Cd toxicity (Goering and Klaassen, 1983, 1984).

Zinc has a major impact on cadmium toxicity. The induction of MT in animals following exposure to low doses of transition metal ions, such as zinc, has been shown to result in protection against acute metal toxicity (Liu *et al.*, 1990). In addition to induction of MT, the presence of zinc concurrent with cadmium dosing can also protect cells from cadmium toxicity by competitive inhibition of transport processes, resulting in less cellular uptake of cadmium. It is important to understand these relationships when undertaking a risk assessment of cadmium.

All vertebrates contain two or more distinct MT isoforms, designated MT I through MT IV (Moffatt and Denizeau, 1997). In mammals, MT I and MT II are present in all tissues (Searle *et al.*, 1984; Hamer, 1986) whereas MT III is expressed mainly in the brain (Palmiter *et al.*, 1992) and MT IV is most abundant in certain stratified squamous epithelium (Quaife *et al.*, 1994). The absence of MT I and MT II has been shown to increase inorganic Cd-induced lethality and

hepatotoxicity, whereas gene over-expression causing enhanced protein production is associated with protection (Klaassen and Liu, 1998). Different genes encode each of these isoforms. The expression of some of these genes is under separate control and may serve different biological purposes (Sadhu and Gedamu, 1988; Jahroudi *et al.,* 1990). The coding and translated amino acid sequences for MT I and MT II exhibit differences in amino acid composition between the two isoforms.

Synthesis of hepatic MT is induced by a number of metals, cytokines and stress hormones, as well as by a wide range of chemicals that act indirectly via a stress or inflammatory response (Hamer, 1986; Bremner, 1987). Numerous studies have shown the ability of Cd to displace Zn from MT and to induce the synthesis of MT (Kagi, 1993). A hallmark of the MT I and MT II genes in particular is their transcriptional induction by Zn and Cd (Andrews, 1990). MT I and MT II are also induced by a variety of stress conditions and compounds, including glucocorticoids, cytokines, reactive oxygen species and other metal ions (Kagi, 1991). However, metal ions such as Cd and Zn are the most potent inducers of MT I and MT II. The high metal-inducibility of MT I and MT II has been linked with their role in heavy-metal detoxification (Moffatt and Denizeau, 1997). MT III and MT IV are unresponsive to these inducers, suggesting that they may play other roles than metal detoxification.

Both MT I and MT II genes have been shown to be coordinately regulated in the mouse and their protein products are thought to be functionally equivalent (Searle *et al.*, 1984; Kagi, 1993). However, discrepancies between tissue levels of MT mRNA and MT protein have been widely reported (Andersen *et al.*, 1983; Lehman-McKeeman *et al.*, 1988; Iijima *et al.*, 1990; Paynter *et al.*, 1990; McCormick *et al.*, 1991; Misra *et al.*, 1997; Carginal *et al.*, 1998). Unfortunately, in some cases, the individual MT isoforms and/or their associated mRNAs were not examined.

Objective

The overall objective of the research effort was the development of reliable techniques to use *in vitro* cellular toxicity testing data to accurately predict toxicological responses in animals. More specifically, the purpose of this effort was to utilize a known toxic chemical (cadmium) to develop an understanding of the relationships between chemical kinetics (rates of chemical movement into the body) and cellular dynamics (cellular response to chemical entering the cells) in order to predict early target organ toxicity. The complete experimental plan for this research is shown in Appendix A.

This report presents data gathered using early cadmium-induced liver toxicity to investigate a cellular-to-animal extrapolation hypothesis and shows how these data could be used, along with BBK modeling, to predict *in vivo* toxicity from *in vitro* data. The research focuses on *in vivo* and *in vitro* components of the toxicological process - chemical kinetics in the animal and cellular responses - in order to facilitate refinement and validation of computer mathematical models (BBK) to describe liver toxicity caused by the model chemical, cadmium.

The purpose of the *in vivo* time course study was to determine the short term (24 hour) kinetics of cadmium, as well as document the effects of this metal on liver enzymes and histology. It was also a goal of the *in vivo* study to determine if two metallothionein isoforms (MT I and MT II) are coordinately regulated. The *in vitro* study was designed to determine if the *in vivo* study results could be predicted using isolated hepatocytes. The chemical kinetics component was derived from Fischer 344 rat tail-vein dosing studies. For the *in vitro* studies, a short cadmium exposure time of 2 hours was used to represent a realistic short-term occupational exposure

scenario. Time course data of cadmium and metallothionein mRNA concentrations in various tissues, plasma and urine and biochemical analyses were gathered following cadmium exposure. The cellular response component was derived from studies using isolated rat liver cells to determine if this *in vitro* method could predict the response and time course of liver toxicity. These components were accomplished with the intent of using computer mathematical modeling techniques to predict the time course of toxicity responses in the rat.

METHODS

Young adult male Fisher 344 rats (230-275 g) were obtained from Charles River Laboratories (Raleigh, NC). Rats were housed individually with each lot segregated from all other rodents for a 7-10 day quarantine/acclimation period. All animals were observed at least twice daily for general health status and any signs of illness. A polycarbonate shoebox caging system with cellulose fiber contact bedding (Cell-Sorb Plus, A.W. Products, Inc., New Philadelphia, OH) was the housing unit during quarantine and prior to the start of the study. Rats were changed into freshly bedded and sanitized cages at least once per week. Rodent chow (#5008, Purina Mills, Inc., St. Louis, MO) and fresh conditioned (reverse osmosis) water was available *ad libitum*. All animals were kept in sanitized animal holding rooms designed to provide 10-15 complete fresh air changes per hour. Room air temperature and humidity were maintained between 21-26°C and 30-70%, respectively. Rodent cage racks remained inside Bio-Clean mass air displacement units, which provided a constant supply of HEPA filtered air. Electronically controlled full spectrum fluorescent light was provided on a 12:12 hour light:dark cycle.

In Vivo Time Course Kinetics and Liver Effects of Cadmium Exposure

Five groups of rats, 36 rats per group, were housed in individual metabolism cages for a 3 day acclimation period prior to dosing. Each rat was dosed intravenously in the lateral tail vein with 0.0, 0.5, 1.0, 2.0 or 3.0 mg/kg Cd, prepared with cadmium acetate (Sigma Chemical Co., St. Louis, MO) in 0.5 mL sterile physiological saline (Medical Supply, Fairborn, OH). The rats were returned to metabolic cages and urine and feces were collected during the course of the study. At 0, 0.5, 1, 3, 6, 9, 12, 18 and 24 hours post dose, four animals per dose group were sacrificed by CO_2 inhalation.

Blood was drawn via the inferior vena cava; plasma and red blood cells were stored for cadmium and metallothionein analyses. Plasma was assayed for ALT and AST activities as markers of cadmium induced hepatic toxicity. Livers were perfused with 0.1 M Tris-acetate buffer (pH 7.4) and a small lobe was removed and fixed in 10% buffered formalin for pathological evaluation to determine the extent of liver necrosis. The remaining liver and all other tissues were flash frozen in liquid nitrogen and stored at -80°C until processing for Cd analysis and MT-mRNA determination. The kidneys were also sampled for Cd analysis.

Determination of Cd Concentration in Plasma and Tissue Samples

Flame atomic absorption spectrophotometry (AAS) was used for analysis of Cd concentrations in serum and tissue samples. In this method, the sample solution is nebulized (converted into fine aerosol) and introduced to the flame as a vaporized and atomized solution. The free atoms are detected in the aerosolized stream by absorption of light as the free atoms move from the ground state to an excited state. The amount of light absorbed is proportional to the

concentration of Cd in the sample.

Before AAS analysis, serum samples from each treatment group were filtered using a 1 mL syringe and a 0.45 μm pore 13 mm filter unit (Millex–HV_{13}, Millipore Corporation, Bedford, MA). Samples were then diluted 1:10 in 0.5% nitric acid.

For tissue preparation, 1 g of wet tissue was homogenized (K43 Homogenizer, TRI-R Instruments, Rockville Centre, NY) in 3 mL of 70% nitric acid and digested to complete dryness in a vacuum oven (Model 5831, National Appliance Company, Winchester, VA) at 110°C. The digestate was dissolved in 3 mL of 0.5% nitric acid and filtered using the same equipment as for serum samples. The digested tissue samples were then diluted again with 0.5% nitric acid, depending on the expected range of Cd tissue concentrations (e.g., a 1:10 dilution, resulting in a final dilution of 30x, for livers treated *in vivo* with 100 μM Cd).

Filtered samples were analyzed on an atomic absorption spectrometer (GBC Scientific Equipment Inc., Arlington Heights, IL) using the "cadmium by flame" method. Cadmium standards, 0.05, 0.5, 1.0, 1.5, 2.5 and 5.0 ppm made from 1000 mg/L (1000 ppm) cadmium standard solution (Environmental Resource Associates, Arvada, CO), were analyzed prior to the samples to establish a standard curve for data analysis.

Serum Liver Enzyme Analyses

The Dimension® clinical chemistry system (Dade Behring, Inc., Deerfield, IL) was used to quantitate aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in rat serum. The methods for these analyses are detailed in Appendix B.

Histopathology

Liver tissues from animals in all dose groups were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin. Each liver slide was graded for vacuolar degeneration, necrosis of hepatocytes, periportal inflammation and random inflammation. Lesions were coded so that lower numbers reflect lower severity of the lesion: 1=minimal, 2=mild, 3=moderate, 4=marked, 5=severe. A score of 0.5 was also used to designate scattered single cell necrosis seen in some animals. Severity of inflammation was also graded; however, inflammation that was interpreted as being secondary to necrosis was not separately graded.

Rat Metallothionein Isoform Regulation *In Vivo*

From the kinetic study, rat tissues were utilized from the 0.5, 1.0 or 2.0 mg/kg Cd exposure groups. These rats were sacrificed by CO_2 inhalation at 0, 0.5, 1, 3, 6, 9, 12, 18 and 24 hours post dose. In addition, sham rats were intravenously dosed in the lateral tail vein with Cd-free saline. Groups of 4 rats were then sacrificed by CO_2 inhalation at 1, 5, 9, 13, 17 and 21 hours after dosing.

RNA Isolation from Liver Tissue

Total RNA was isolated from rat livers using the RNeasy® Mini Kit (Qiagen, Inc., Valencia, CA). The RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues was followed with minor modifications. RNA lysis Buffer RLT was prepared by adding 10 μL β-mercaptoethanol (β-ME) per 1 mL of RNeasy Lysis Buffer (Buffer RLT) in a fume hood. Approximately 30 mg of liver tissue was added to 600 μL of RNA lysis Buffer RLT in a 2 mL collection tube. A stainless steel bead was added to each 2 mL tube containing the RNA lysis Buffer RLT and liver tissue. The tissue was homogenized on a Mixer Mill MM 300 (F. Kurt Retsch GmgH & Co. KG, Haan, Germany) for 2 minutes at 20 Hz. The Mixer Mill rack was rotated to allow even homogenization and homogenized for another 2 minutes at 20 Hz. The tissue sample (including the bead) was centrifuged for 3 minutes at 15,000 rpm in a Micromax microcentrifuge (International Equipment Co., Needham Heights, MA).

The supernatant was carefully transferred to a 2 mL collection tube and 600 μL of 70% ethanol was added. Each tube was mixed well by pipetting. Next, 700 μL of sample was added to a RNeasy mini column placed in a 2 mL collection tube. The tube was gently closed and centrifuged for 25 seconds at 15,000 rpm. The flow-through was decanted and the column blotted. Decanting and blotting was repeated until the entire sample was processed. To wash the column, 700 μL of Buffer RW1 was applied to the column and the column was centrifuged for 25 seconds at 15,000 rpm. The flow-through was discarded and the Buffer RW1 wash was performed again. After the second Buffer RW1 wash, the flow-through was discarded and the RNeasy column was transferred to a new 2 mL collection tube. Another wash was performed by adding 500 μL Buffer RPE onto the RNeasy column and centrifuging for 25 seconds at 15,000 rpm. The flow-through was discarded and another 500 μL Buffer RPE was added to the column; it was centrifuged 2 minutes 15 seconds at 15,000 rpm, and the flow-through discarded. To dry the column and prevent any chance of Buffer RPE carryover, the column was centrifuged another 1 minute 15 seconds at 15,000 rpm.

To elute the RNA, the RNeasy column was transferred to a new 1.5 mL collection tube and 30 μL RNase-free water was pipetted directly onto the RNeasy silicia-gel membrane. The RNeasy column was allowed to sit at room temperature for 1 minute and centrifuged for 2 minutes 15 seconds at 15,000 rpm. This step was performed twice and the eluates were combined and placed on ice. A 1:50 dilution was made in a 1.5 mL tube by adding 2 μL total RNA extract to 98 μL 1X TE. The sample was vortexed and the concentration of total RNA was determined by measuring the absorbance at 260 nm (A260) in a GeneQuant Pro spectrophotometer (Biochrom Ltd., Cambridge, England). The purity of the total RNA was determined by the ratio (A260/A280). A ratio of 1.9-2.1 indicated a pure RNA sample. The integrity of the RNA was determined after separation by agarose gel electrophoresis by illuminating the 28S and 18S ribosomal RNA bands with ethidium bromide (Figure 1). The bands were viewed by UV light using the Fisher Biotech Electrophoresis System Transilluminator (Fisher Scientific, Pittsburgh, PA). The RNA samples were aliquoted into 5 μg aliquots and stored in the -80°C freezer until processed to cDNA.

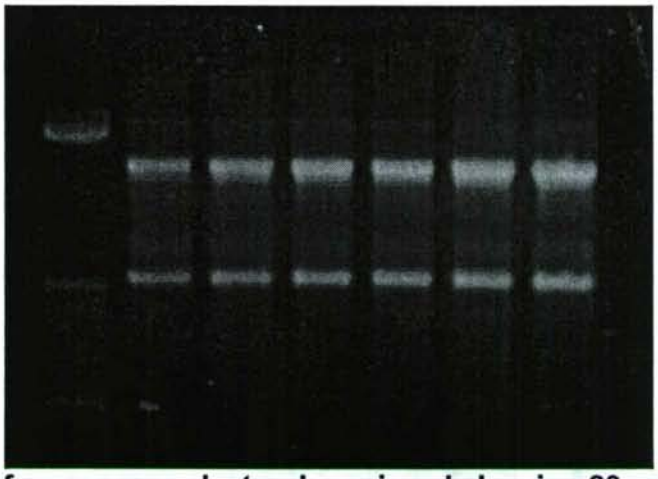

Figure 1. Example of an agarose electrophoresis gel showing 28s and 18s rRNA bands.

First Strand Synthesis of cDNA by Reverse Transcription

First strand synthesis of cDNA from total RNA was performed using the 5 µg total RNA aliquots and hepatocytes. All of the reagents in this procedure were thawed on ice and quick-spun (except the enzyme). To each 5 µg sample, 1 µL 100 pmol/µL oligo dt-T7 promoter and the appropriate volume of DEPC water was added to obtain a final reaction volume of 25 µL. Each sample was vortexed (Genie 2, Fisher Scientific, Bohemia, NY) and quick-spun using a Stratagene Profuge® 10K (La Jolla, CA). The samples were then incubated for 10 minutes at 70°C in a GeneAmp® 9700 polymerase chain reaction (PCR) System (Applied Biosystems, Foster, CA).

During the 10 minute incubation, the master mix was prepared by combining 5 µL 5X 1st strand buffer (Invitrogen, Carlsbad, CA), 2 µL 0.1 M DTT (Invitrogen, Carlsbad, CA), and 1 µL 10 mM dNTP mix (Invitrogen, Carlsbad, CA) for each sample. After the 10 minute 70°C incubation, the samples were immediately put on ice, vortexed and quick-spun. The samples were incubated for 2 minutes at 42°C in a Model 180 Molecular Biology Water Bath (Precision Scientific, Chicago, IL). After the 2-minute incubation, 8 µL of the master mix was added to each sample. The samples were mixed and incubated another 2 minutes in the 42°C water bath. Following this 2-minute incubation, 1 µL Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) was added to each sample and the samples mixed thoroughly. The samples were incubated for 1.5 hours at 42°C in the Gene Amp® 9700 PCR system. Once the incubation was completed the samples were immediately placed on ice, vortexed, quick-spun and stored in the -20°C freezer until used in Real-Time PCR.

Real-Time Polymerase Chain Reaction

Analysis: Real-Time PCR was performed on cDNA samples to quantitate the amount of MT mRNA in Cd treated liver tissue and hepatocytes. Primer Express® software (Applied Biosystems, Foster City, CA) was used to design the primers. The primers used were rat specific MT I (forward primer: 5'-AAT GTG CCC AGG GCT GTG T-3' and reverse primer: 5'-CGT CAC TTC AGG CAC AGC A-3'); rat specific MT II (forward primer: 5'- AAG TGC TCC CAG GGC TGC-3' and reverse primer: 5'-CCC CAC TTC AGG CGC A-3'); and GAPDH

(forward primer: 5'-GAT TCT ACC CAC GGC AAG TTC A-3' and reverse primer: 5'-GGT TTC CCA TTG ATG ACC AGC T-3') (Integrated DNA Technologies, Inc., Coralville, IA). All primers were reconstituted in 1X TE buffer to create a stock solution of 1 mM. A 1:20 dilution (5 µL stock solution to 95 µL DEPC) was made to create a working stock concentration of 50 mM. SYBR® Green PCR Master Mix (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, dNTPs (with dUTP), Passive Reference 1, and optimized buffer components (proprietary formulation)) (Applied Biosystems, Foster City, CA) and AmpErase® UNG (Uracil N-glycoslyase) was used in all Real-Time PCR reactions. The SYBR® Green I dye incorporation into a real-time PCR reaction allows the detection of any double-stranded DNA generated during PCR. Target specific probes are not required; however, both specific and non-specific products will generate a signal. The incorporation of the hot-start enzyme AmpliTaq Gold® DNA Polymerase minimizes non-specific product formation.

A master mix was made by combining the appropriate volume of SYBR® Green PCR Master Mix, AmpErase® UNG, DEPC water, Forward Primer and Reverse Primer as shown in Table 1. The template (cDNA) was added to each appropriate well. To each well of a MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA), 23 µL of the corresponding master mix (MT I, MT II, GAPDH) was added. All MT I cDNA samples were diluted 1:100 (1 µL cDNA to 99 µL TE buffer). To each well, 2 µL of the appropriate template (cDNA) was added to equal a total reaction volume of 25 µL. MicroAmp Optical Caps (Applied Biosystems, Foster City, CA) were applied to the reaction plate. The reaction plate was run on the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the thermal cycling conditions in Table 2. On the disassociation curve, the melting temperature (Tm) of GAPDH was 80° C, MT I was 83.8°C, and MT II was 83.4°C.

Table 1. Protocol for using SYBR® Green PCR Master Mix

Reagents	Volume/25 µL Reaction (µL)	# of Samples	Sample Volume (µL)
2X SYBR Green PCR Master Mix	12.5	106	1325.0
AmpErase UNG (1U/ µL)	0.25	106	26.50
Water	8.25	106	874.50
Primer Forward	1	106	106.0
Primer Reverse	1	106	106.0
Template (cDNA)	2	106	212.0
Total volume			2650.0
Total volume including Primers & cDNA	25	106	2650.0
Tube 1 (Add MT I F&R 41 µL)	861		
Tube 2 (Add MT II F&R 41 µL)	861		
Tube 3 (Add GAPDH F&R 24 µL)	504		
Total volume without Primers & cDNA	2226		

Table 2. Real-Time PCR thermal cycle conditions

Step	AmpErase UNG Incubation*	AmpliTaq Gold Activation*	PCR		Dissociation Curve Profile		
	HOLD	HOLD	Cycle (40 cycles)		HOLD		
			Denature	Anneal/ Extend			
Temp	50°C	95°C	95°C	60°C	95°C	60°C	95°C
Time	2 min	10 min	15 sec	1 min	15 sec	20 sec	15 sec
Volume	25 µL						

*Required for optimal activity of those enzymes

All plates contained no template controls (NTC) and no amplification controls (NAC). All samples were run in triplicate except for the NTC which were run in duplicate. All reaction plates were stored in the -20°C freezer.

Validation: Prior to performing Real-Time PCR analysis, a validation experiment was utilized to demonstrate that the PCR efficiencies of the target mRNA (MT I and MT II) and endogenous reference mRNA (GAPDH) were approximately equal. A sensitive method for assessing PCR efficiency is to determine how ΔC_T (threshold cycle) varies with template dilution. The absolute value of the slope of the ΔC_T ($C_{T\ (target)} - C_{T\ (reference)}$) versus log RNA input should be less than 0.1.

The total RNA and cDNA samples used in this study were from untreated rat liver tissue and untreated hepatocytes. The total RNA isolation and cDNA synthesis was performed as described above. Two-fold serial dilutions (1, 2, 4, 8, 16, 32, 64 and 128) were made using TE buffer and a 5 µg total RNA sample.

Calculation: The comparative C_T method uses a mathematical formula ($2^{-\Delta\Delta C_T}$) to calculate the amount of target normalized to an endogenous reference and relative to a calibrator. This method was used to determine relative quantitation. C_T values for MT I and MT II amplification were normalized by subtracting the C_T values for GAPDH using the equation ($C_{T\ (MT\ I\ or\ MT\ II)} - C_{T\ (GAPDH)} = \Delta C_T$). The ΔC_T for the control liver (1 hour time point/0 mg/kg dose) was subtracted from the ΔC_T for Cd-treated liver to calculate the fold change in MT I and MT II expression ($\Delta C_{T\ (Cd\text{-}treated)} - \Delta C_{T\ (control)}$) = $\Delta\Delta C_T$. This represents the fold change in MT I and MT II mRNA expression.

Statistical Analysis: The treatment groups of MT I and MT II mRNA expression were compared by a multivariate analysis (MANOVA) with a total N of 108. Means were compared using the Tukey comparisons with a Type I error level of $p<0.05$.

Rat Metallothionein Isoform Regulation *In Vitro*

Rat liver perfusion surgery was performed to allow isolation and culture of primary hepatocytes. Briefly, the rat was anesthetized with CO_2 and the abdominal and thoracic cavities of the anesthetized animal were exposed. Loose ligatures of suture were then placed around the superior vena cava above the diaphragm and around the portal vein approximately 5 mm from the liver (between liver and gastro-intestinal vein). A small incision in the portal vein was then made below the ligature. The first step of the antigrade two-step perfusion method was accomplished by cannulation of the portal vein followed by clamping of the posterior vena cava anterior to the diaphragm. Flow of calcium-free perfusion buffer was then activated (20 mL/min) immediately followed by cutting of the posterior vena cava (anterior to the renal vein) to allow drainage of the perfusion buffer. Following complete removal of blood and calcium from the liver, the second step of the liver perfusion was initiated by continuous perfusion with digestion buffer containing collagenase and calcium for 20 min to digest the liver connective tissue.

Viable primary rat hepatocytes were enriched from the crude hepatocyte suspension by low speed centrifugation for 3 min, repeated three times. Enriched hepatocytes were seeded in 6-well (1.2×10^6 cells/well) culture plates previously coated with rat tail collagen (Upstate Biotechnology, Lake Placid, NY) at 2.6 mg/cm^2. After 3 hours of incubation in a 95% air/5% CO_2 atmosphere at 37°C to allow for attachment, rat hepatocytes were re-fed with 2.0 mL of Chee culture medium lacking dexamethasone.

Primary hepatocytes were exposed to Cd at four doses (0 µM, 5 µM, 10 µM and 15 µM) in the presence of albumin for 2 hours at 37°C. The 5 µM dose was below the maximum tolerated dose (MTD, as determined by MTT analysis below), the 10 µM was at the MTD and the 15 µM dose was at the effective concentration that resulted in 20% toxicity (EC_{20}). At -2, 0, 1, 4, 7, 10, 16 and 22 hours, cell samples were pooled.

Cell viability and cytotoxicity was determined prior to pooling using the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazan) by the mitochondrial enzyme succinate-dehydrogenase, which requires NADH. This reduction only takes place in cells that maintain normal metabolic function. The amount of formazan produced is proportional to the number and viability of cells present. Therefore, this is an indirect measure of cytotoxicity and indicator of mitochondrial functional integrity plus a simple colorimetric method of cellular viability. The color change was measured spectrophotometrically in the SpectraMAX 190 plate reader (Molecular Devices, Inc, Sunnyvale, CA).

The MTT stock solution (5 mg/mL) was diluted 1:10 in Chee media supplemented with ITS (insulin/transferrin/sodium selenite). The media from each well was aspirated and 1 mL of MTT working solution was added to each well. The plate was incubated for 15 minutes at 37°C in the CO_2 incubator. After the incubation, the MTT working solution was aspirated from the wells and 1 mL of acidified isopropanol was added to each well to extract the reaction product from the cells. The plate was placed on the RotoMix Type 50800 rotator (Thermolyne, Dubuque, IA) for 10 minutes. To each well of a 96-well plate, 200 µL of supernatant was added and the 96-well plate was read spectrophotometrically in the SpectraMAX micro plate reader.

Dose-response curves of the MTT data were generated using SigmaPlot software (Systat Software, Inc., Richmond, CA). The EC_{50} concentrations and MTD were determined from these curves.

RNA Isolation from Hepatocytes

Total RNA was isolated from rat hepatocytes using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA). The RNeasy Mini Protocol for Isolation of Total RNA from Animal Cells was followed with minor modifications. RNA lysis Buffer RLT was prepared by adding 10 µL β-mercaptoethanol (β-ME) per 1 mL of RNeasy Lysis Buffer (Buffer RLT) in a fume hood. To each well of a 6-well plate, 600 µL of RLT lysis buffer was added. Each well was scraped with the large end of a pipette tip for approximately 45-60 seconds to loosen the cells. The sample from each well was homogenized using the QIAshredder. One shredder tube was used for each dose and one 1.5 mL tube was used for each well (i.e., the same dose was in two wells; one shredder tube and two 1.5 mL tubes were used). To each 1.5 mL tube, 600 µL of 70% ethanol was added. From each well, 600 µL of sample was added to the shredder tube and centrifuged for 2 minutes at 15,000 RPM. After centrifugation the sample was added to 1.5 mL tube containing ethanol and mixed by pipetting. From this point on, the RNA isolation procedure for liver tissue and hepatocytes were identical.

First strand synthesis of cDNA by reverse transcription and real-time polymerase chain reaction methods were identical to those used for liver tissue samples. See these methods in the "Rat Metallothionein Isoform Assays" section above.

RESULTS

In Vivo Time Course Kinetics and Liver Effects of Cadmium Exposure

Time course kinetic data of Cd in serum showed a dose dependent trend in the magnitude of concentration and the shift in serum Cd clearance from a fairly mono-exponential curve for the lower two doses, to a bi- or tri-exponential clearance for the 2.0 and 3.0 mg/kg groups (Figure 2). Also, it was observed that the earliest sampled serum concentrations followed a dose-response pattern, with the 3.0 mg/kg dose group having the highest serum concentration and the 0.5 mg/kg dose group having the lowest concentration. After these early time points, there was a significant drop in serum concentration by the four hour sampling time in the 3.0 mg/kg group, below that of the other dose groups. A similar but not as precipitous a drop was seen in the 2.0 mg/kg dose group in the early time points; the concentration curve then paralleled the 0.5 and 1.0 mg/kg dose groups at the later time points.

Average time point data plotted in Figures 2-4 are included in Appendix C. Note that the 0 and 6 hour time point serum Cd concentrations for the control rats were reported as zero and therefore not plotted on the logarithmic scale in Figure 2. These serum samples were not analyzed due to technical difficulties.

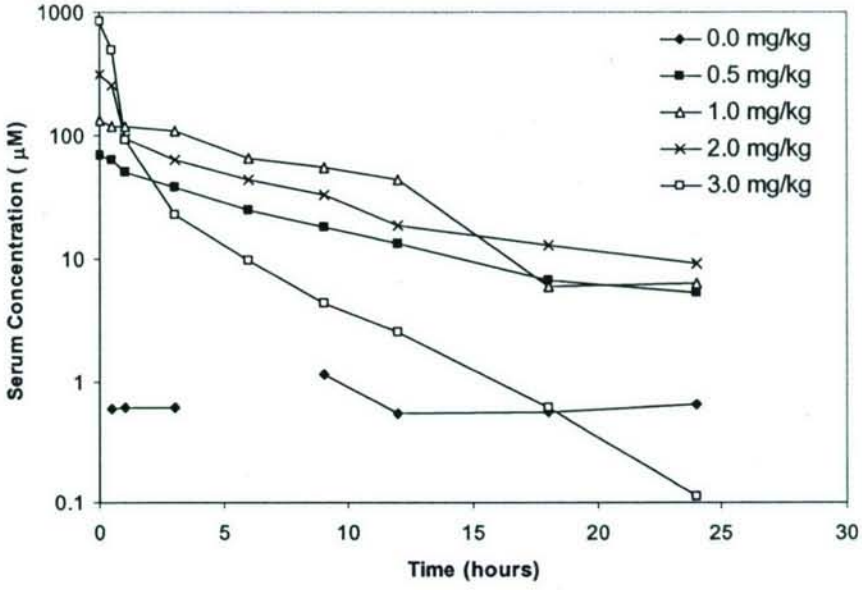

Figure 2. Time course serum Cd concentrations in male F344 rats following *iv* administration of 0, 0.5, 1.0, 2.0 or 3.0 mg Cd/kg bodyweight

Evidence of these unique serum Cd clearance patterns were partially reflective of what was occurring in the time-course of Cd concentration in the liver and kidney tissues (Figures 3 and 4, respectively). The Cd concentration in liver was slightly higher for the 2.0 mg/kg dose group until the 3 hour time point, at which time the liver concentration for the 3.0 mg/kg group continued to rise well above that of the 2.0 mg/kg group and all other dose groups, as would be hypothesized. The lower liver concentration value for the 3.0 mg/kg dose group was not what would be predicted based on normal dose response patterns.

While the concentration of Cd in the kidney tissue was highest in the 3.0 mg/kg dose group, there was an indication as early as 0.5 hours after dosing that the kidney was also exhibiting different Cd kinetics at the 3.0 mg/kg dose and possibly the 2.0 mg/kg dose; these time course curves were indicative of significant changes in kinetics of Cd at these two higher doses. Both the liver and kidney tissues exhibited an increase in tissue concentration with increasing time, out to the 24 hour sacrifice time point. This suggests distribution of the Cd from the general circulation and tissues with less affinity for Cd to the liver and kidney, two tissues known to have a high affinity for Cd.

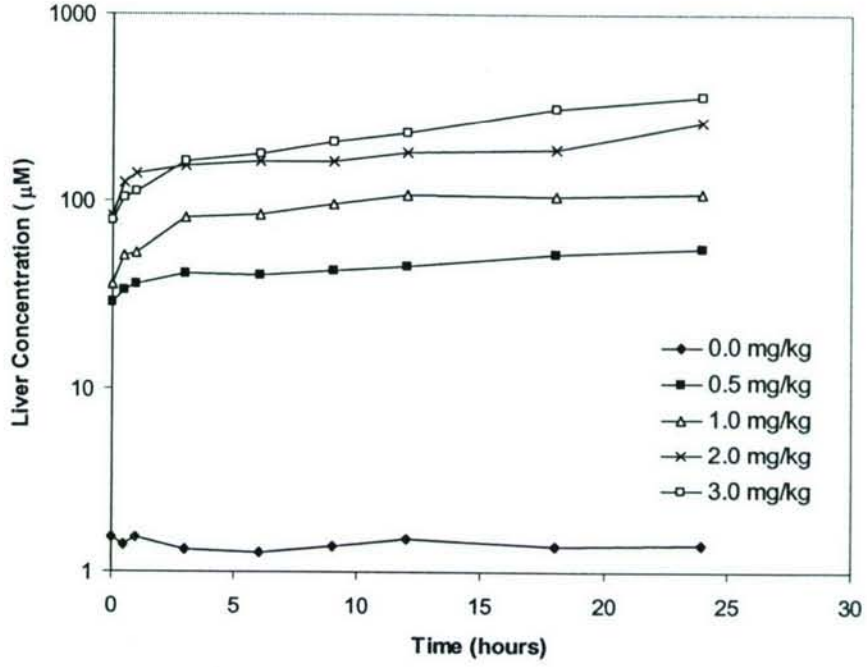

Figure 3. Time course liver Cd concentrations in male F344 rats following *iv* administration of 0, 0.5, 1.0, 2.0 or 3.0 mg Cd/kg bodyweight

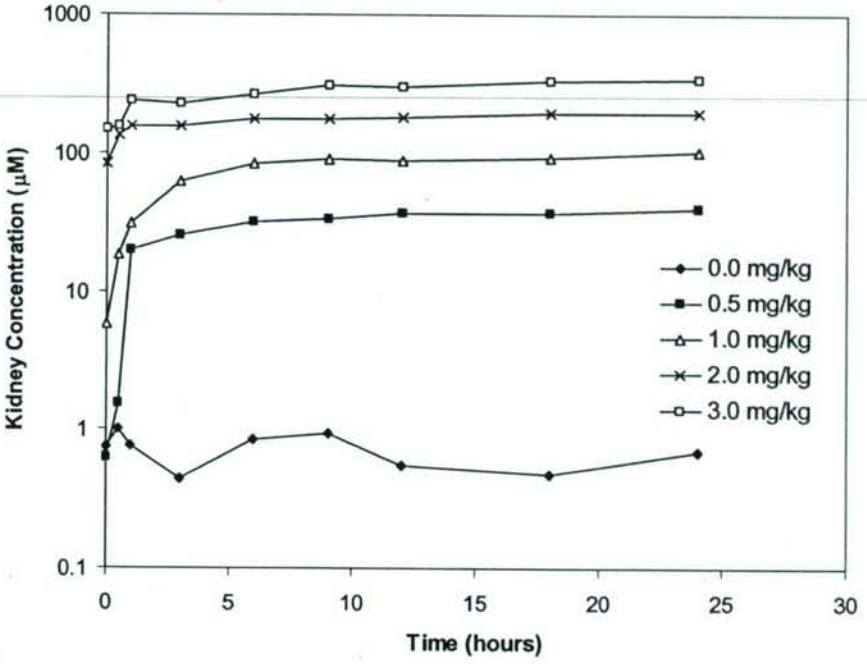

Figure 4. Time course kidney Cd concentrations in male F344 rats following iv administration of 0, 0.5, 1.0, 2.0 or 3.0 mg Cd/kg bodyweight

Serum Liver Enzymes (AST and ALT)

The serum enzymes AST and ALT were assayed as measures of toxic damage to liver tissues from acute Cd exposure. ALT is an enzyme which predominately appears in liver cells, with lesser amounts in the kidneys, heart and skeletal muscles; ALT is a relatively specific indicator of acute liver cell damage. When such damage occurs, ALT is released from the liver cells into the bloodstream, often before jaundice appears, resulting in abnormally high serum levels that may not return to normal for days or weeks. Measurement of both ALT and AST helps distinguish between myocardial (heart) and liver tissue damage. AST levels fluctuate in response to the extent of cellular necrosis (cell death) and therefore are elevated during the most acute phase of tissue damage, including damage to either the liver or the heart.

In this study, the dose dependent magnitude and the time-course of these two enzymes was virtually identical (Figure 5 and 6). The samples from the 3.0 mg/kg dose were too high to be properly assayed for both AST and ALT with the experimental technique employed in this study. There was inadequate sample available to provide enough repeated dilution studies to bring the serum samples into range of the assay.

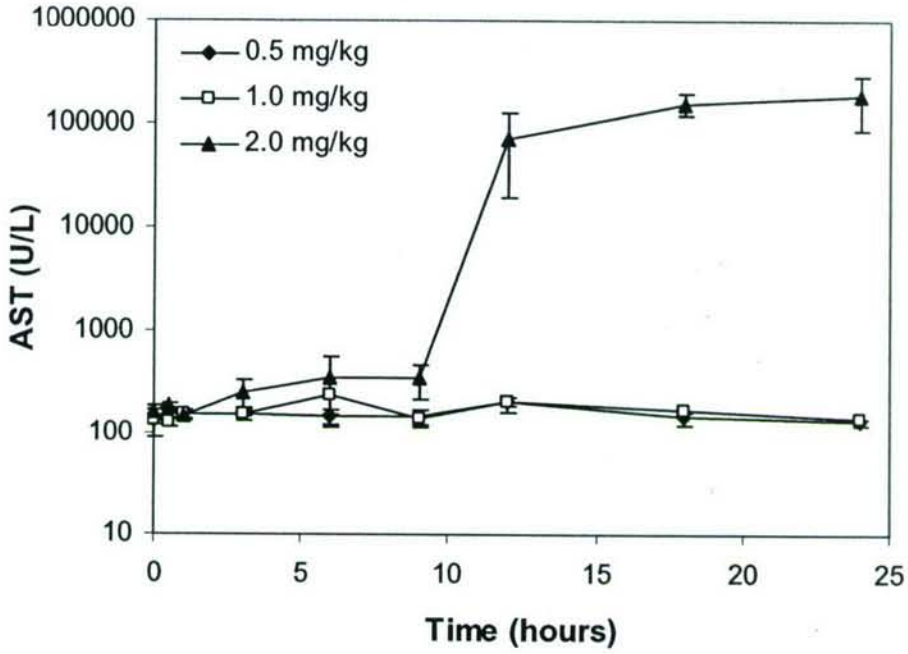

Figure 5. Serum AST concentrations in male F344 rats following *iv* administration of 0.5, 1.0 or 2.0 mg Cd/kg bodyweight

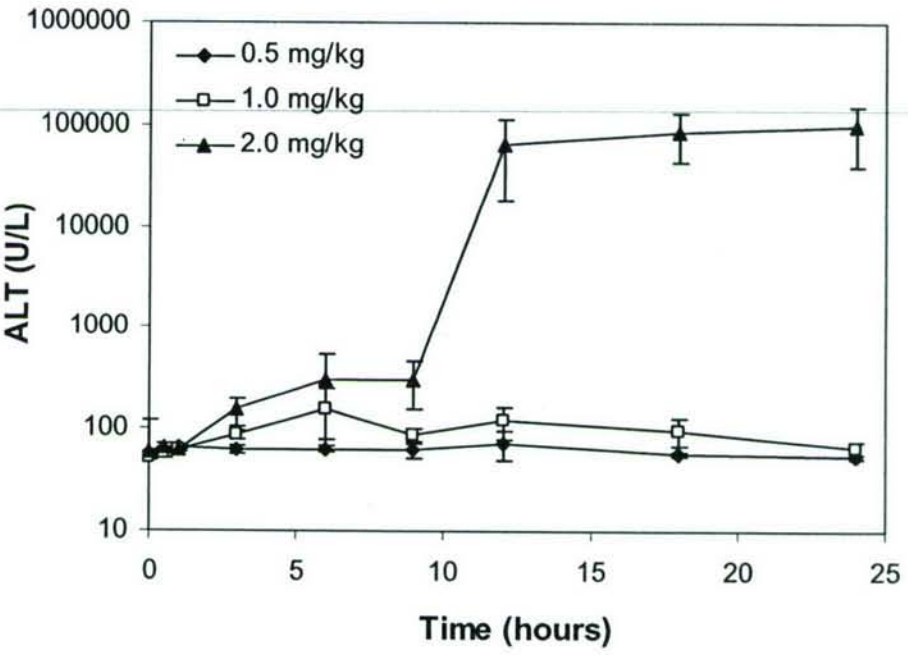

Figure 6. Serum ALT concentrations in male F344 rats following *iv* administration of 0.5, 1.0 or 2.0 mg Cd/kg bodyweight

Histopathological examination was performed on liver tissues from all five dose levels. A dose effect appeared evident in these livers for hepatocellular degeneration and necrosis. The most severe lesions were observed at the latter time points in the two highest dose groups (2.0 and 3.0 mg/kg). Livers from controls and the 0.5 and 1.0 mg/kg groups were either within normal limits or had only mild lesions. Examples of findings are shown in Figures 7 and 8. Magnifications were optimized to demonstrate normal histology versus toxicological effects.

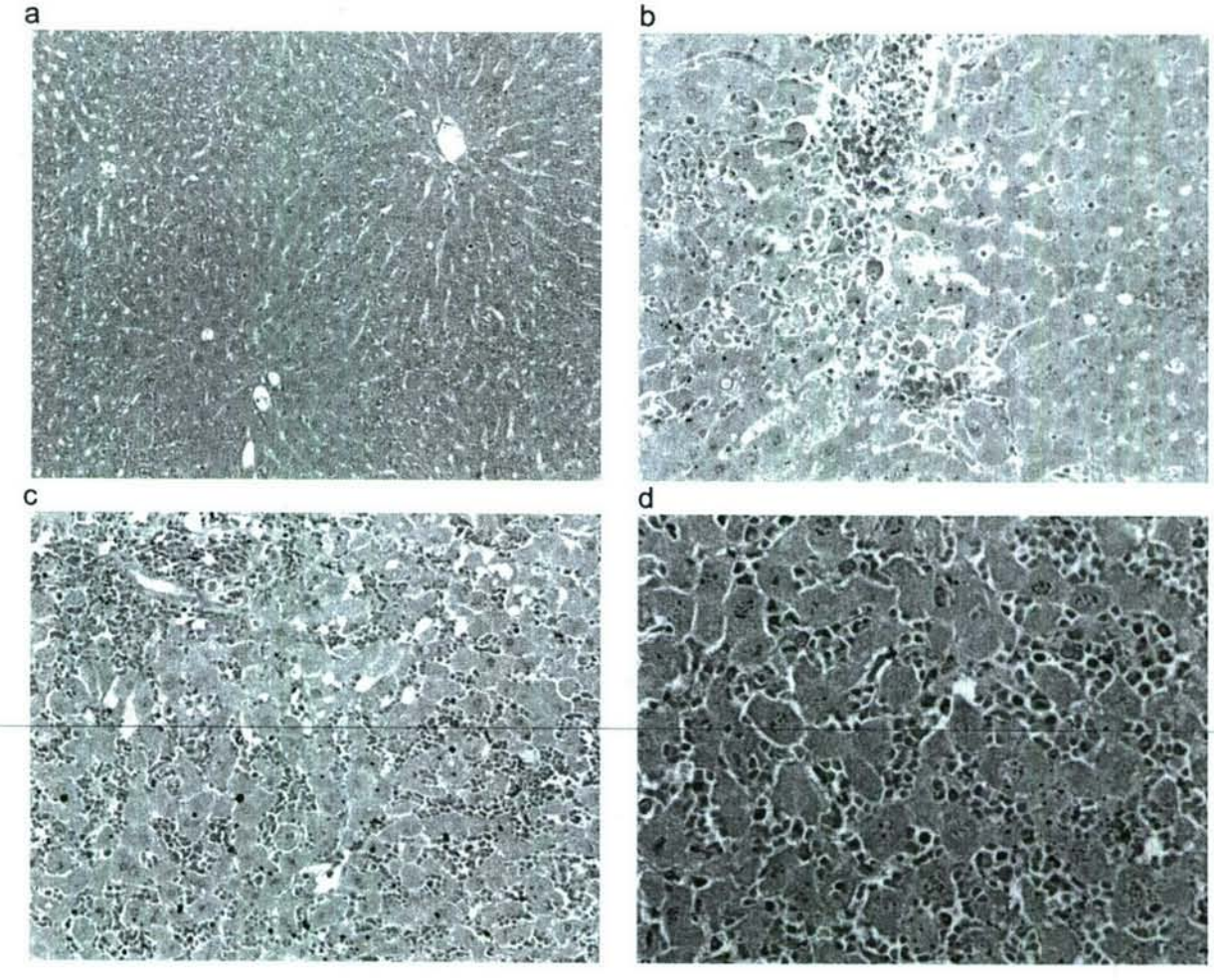

Figure 7. Examples of necrosis and hemorrhage in the livers of F344 rats following histopathological examination

a. Normal liver tissue from control rats (10x magnification). b. Moderate liver necrosis and hemorrhage 0.5 hours following *iv* dosing with 3.0 mg Cd/kg bodyweight (20x). c. Severe necrosis and hemorrhage 12 hours following *iv* dosing with 3.0 mg Cd/kg bodyweight (20x). d. Severely necrotic hepatocytes 12 hours following *iv* dosing with 3.0 mg Cd/kg bodyweight (40x).

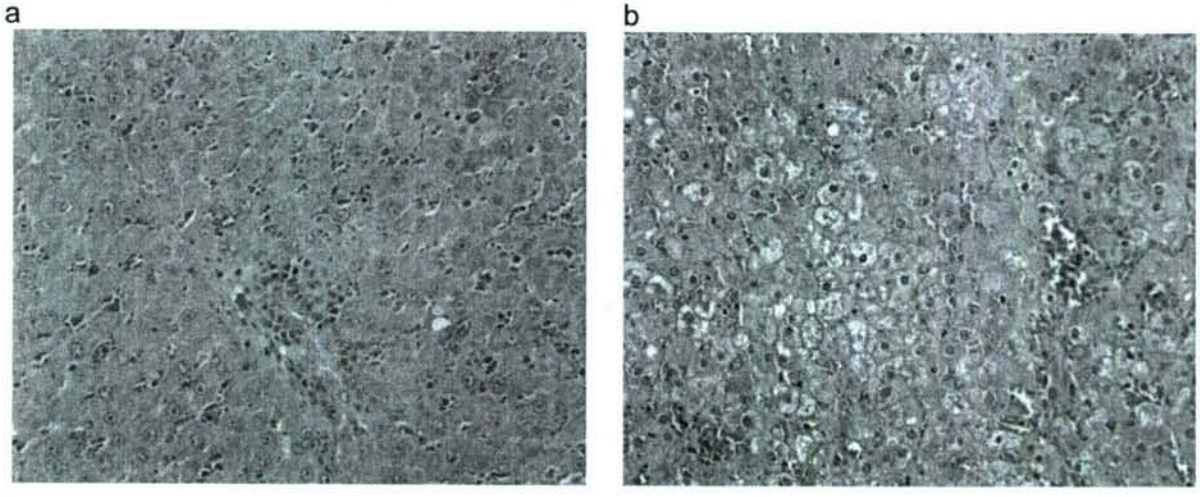

Figure 8. Examples of inflammation and degeneration in the livers of F344 rats following histopathological examination

a. Mild inflammation of the periportal mononuclear cells 18 hours following *iv* dosing with 3.0 mg Cd/kg bodyweight (40x magnification). b. Marked vacuolar degeneration 18 hours following *iv* dosing with 3.0 mg Cd/kg bodyweight (40x).

Rat Metallothionein Isoform Regulation *In Vivo*

Real Time PCR Validation Study

Before the treated liver samples could be analyzed by Real-Time PCR, a validation experiment was performed to demonstrate that the PCR efficiencies of the target and reference were approximately equal. The absolute value of the slope should be less than 0.1. Proving that the efficiencies of both the target and reference rates of transcription were approximately equal allowed the use of the comparative C_T method for quantitation. To determine if the two efficiencies were equal, varying concentrations of total RNA were analyzed by Real Time PCR for MT I, MT II and GAPDH using the forward and reverse primers.

The average MT I C_T values were calculated along with the ΔC_T value (Table 3). The log ng of the total RNA was plotted versus the ΔC_T for MT I (Figure 9). The absolute value of the slope of the log ng total RNA versus the ΔC_T was greater than 0.1. Since MT I did not meet the performance criteria, another experiment was performed. In this experiment the serial dilutions of the total RNA were extended to greater dilutions and MT I was reanalyzed (Table 4). From the plot of the ΔC_T versus the log ng total RNA (Figure 10), the slope was -0.0420 which met the performance criteria. Since MT I required further dilutions to satisfy the performance criteria, all samples that were analyzed for MT I were diluted 1:100.

Table 3. Average C_T values for MT I and GAPDH in rat liver tissue

Dilution	Total RNA (ng)	MT I Average C_T	GAPDH Average C_T	Total RNA (log ng)	ΔC_T (MTI-GAPDH)
1	500	15.6±0.2	14.6±0.1	2.7	1.0±0.3
2	250	16.4±0.6	15.2±0.1	2.4	1.2±0.6
4	125	17.2±0.3	16.0±0.2	2.1	1.3±0.4
8	62.5	18.4±0.3	16.7±0.3	1.8	1.7±0.5
16	31.25	19.7±0.4	17.4±1.2	1.5	2.3±1.2
32	15.63	20.7±0.3	18.8±0.6	1.2	1.9±0.7
64	7.81	21.6±0.4	19.8±0.4	0.9	1.9±0.5
128	3.91	23.6±0.3	20.3±1.0	0.6	3.3±1.1
Slope		-0.8693			
Absolute Value		0.8693			

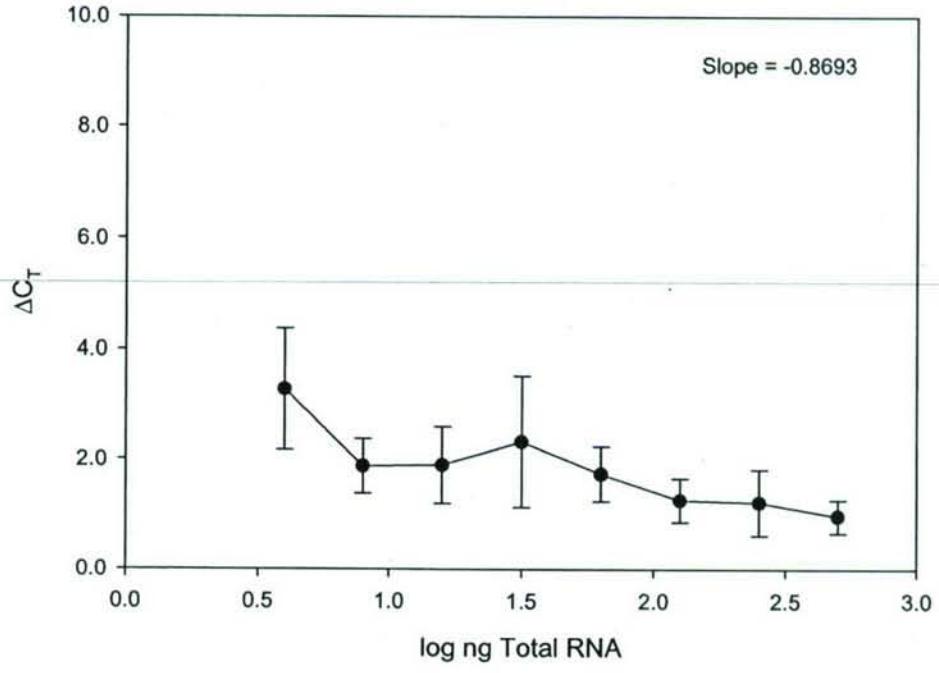

Figure 9. Relative efficiency plot of MT I and GAPDH in rat liver tissue

Table 4. Average C_T values for MT I and GAPDH using additional dilutions of rat liver tissue

Dilution	Total RNA (ng)	MT I Average Ct	GAPDH Average Ct	Total RNA (log ng)	Δ C_T (MTI -GAPDH)
8	62.5	17.0±0.3	15.7±0.1	1.8	1.3±0.4
16	31.25	18.0±0.0	16.5±0.2	1.5	1.5±0.2
32	15.63	18.8±0.2	17.3±0.1	1.2	1.5±0.2
64	7.81	19.9±0.2	18.2±0.2	0.9	1.7±0.3
128	3.91	20.9±0.3	19.2±0.2	0.6	1.7±0.3
256	1.96	21.8±0.5	20.2±0.3	0.3	1.6±0.6
512	0.98	22.6±0.6	21.2±0.1	0.0	1.4±0.6
1024	0.49	23.5±0.3	22.0±0.4	-0.3	1.6±0.4
Slope		-0.0420			
Absolute Value		0.0420			

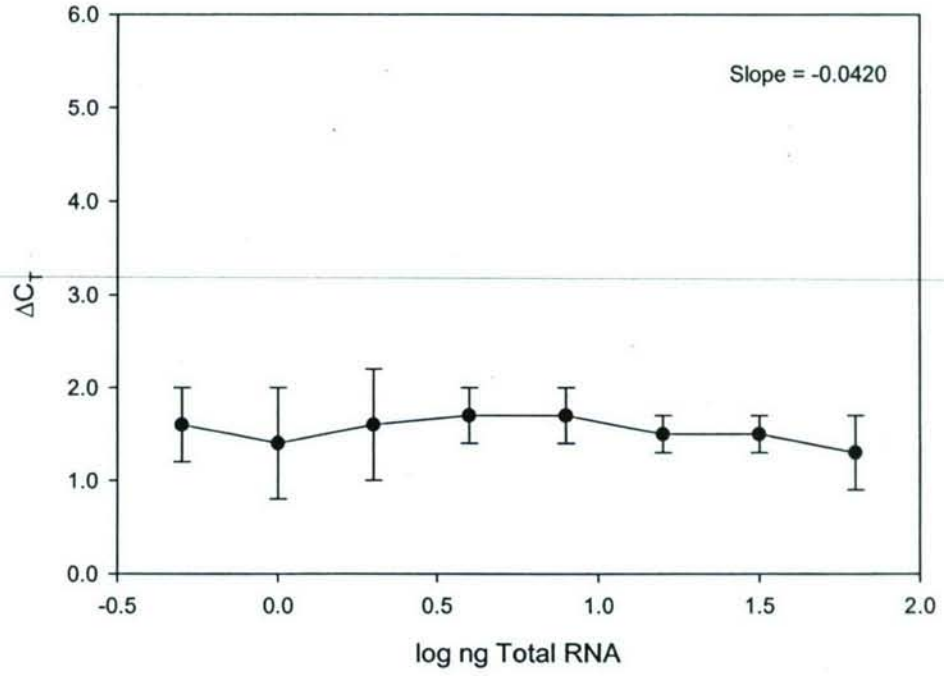

Figure 10. Relative efficiency plot of MT I and GAPDH using additional dilutions of rat liver tissue

MT II was analyzed using the same criteria as in the initial experiment for MT I. The average C_T values for MT II and GAPDH were calculated along with the ΔC_T (Table 5). The log ng total RNA was plotted versus the ΔC_T and the slope was determined to be -0.0981 (Figure 11).

Since the slope was within the acceptable parameter range, no further validation experiments were performed for MT II.

Table 5. Average C_T values for MT II and GAPDH in rat liver tissue

Dilution	Total RNA (ng)	MT II Average C_T	GAPDH Average C_T	Total RNA (log ng)	ΔC_T (MTII -GAPDH)
1	500	16.8±0.2	14.6±0.1	2.7	2.2±0.3
2	250	18.7±0.4	15.2±0.1	2.4	3.5±0.5
4	125	19.0±0.4	16.0±0.2	2.1	3.1±0.5
8	62.5	20.1±0.5	16.7±0.3	1.8	3.4±0.6
16	31.25	21.9±2.5	17.4±1.2	1.5	4.5±0.6
32	15.63	21.8±0.4	18.8±0.6	1.2	3.1±0.7
64	7.81	22.6±0.4	19.8±0.4	0.9	2.8±0.6
128	3.91	23.2±0.7	20.3±1.0	0.6	2.9±1.3
Slope		-0.0981			
Absolute Value		0.0981			

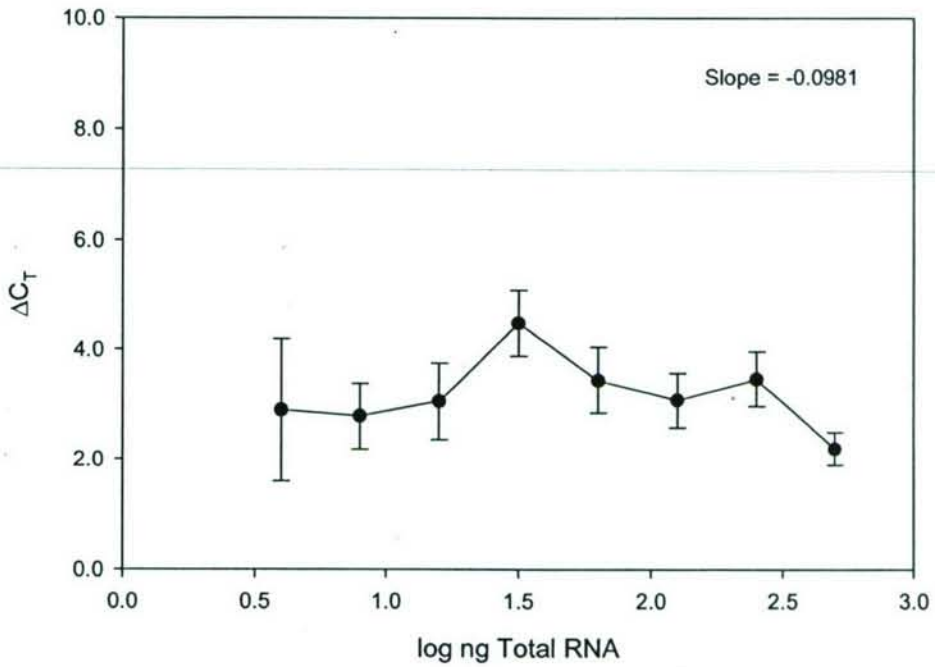

Figure 11. Relative efficiency plot of MT II and GAPDH in rat liver tissue

Effects of Cd on MT I and MT II mRNA Expression *In Vivo*

Real-Time PCR was used to determine the time course of mRNA expression of MT I and MT II after exposure to various doses of Cd acetate (0.0 0.5, 1.0 and 2.0 mg/kg) in F344 rats. The time course data for MT I mRNA expression (Figure 12), indicate that the MT I mRNA levels peaked at 6 hours for all three doses. The 2.0 mg/kg dose peaked at the highest level followed by the 1.0-mg/kg dose and the 0.5 mg/kg dose. Both the 0.5 mg/kg and 1.0 mg/kg doses declined to baseline levels at 12 hours and then started to increase again after 12 hours (Figure 9). The 2.0 mg/kg dose declined slightly at 9 hours but remained elevated until 24 hours. These results indicate a significant effect of both dose and time in MT I mRNA expression ($p<0.0001$). The data for sham controls indicate that either there was a slight diurnal cycle for MT I mRNA levels with levels peaking at 4 p.m. and/or that the sham treatment had a minor effect on MT I mRNA kinetics.

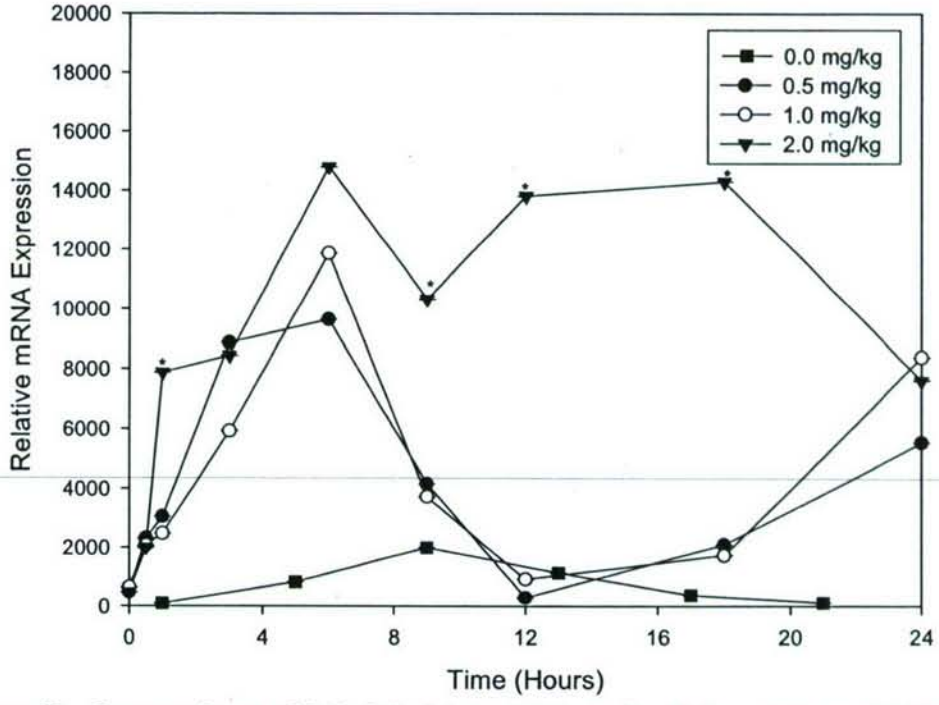

Figure 12. Comparison of 0.0, 0.5, 1.0 and 2.0 mg/kg Cd acetate on MT I mRNA expression

*Indicates significant difference between the means of the 0.5, 1.0 and 2.0 mg/kg doses at a specified time ($p=0.0001$, $p=0.0228$, $p<0.0001$ and $p=0.0109$). Note: Standard error bars were smaller than the symbol and do not show ($n = 4$).

The time course data for MT II mRNA expression exhibited a different pattern than that observed for MT I. For all three doses, a peak in MT II mRNA expression was observed at 1 hour with the 2.0 mg/kg dose peaking the highest. This early peak was followed by a rapid decrease in MT II mRNA expression for all three doses returning to baseline levels at 3 hours (Figure 13). At 9 hours all three doses peaked again. Both the 0.5 mg/kg and 1.0 mg/kg doses declined at 12 hours and then increased again at 24 hours (Figure 13). The 2.0 mg/kg remained elevated out to 24 hours. From these results it was concluded that MT II mRNA expression was significantly affected by both time and dose also ($p<0.0001$).

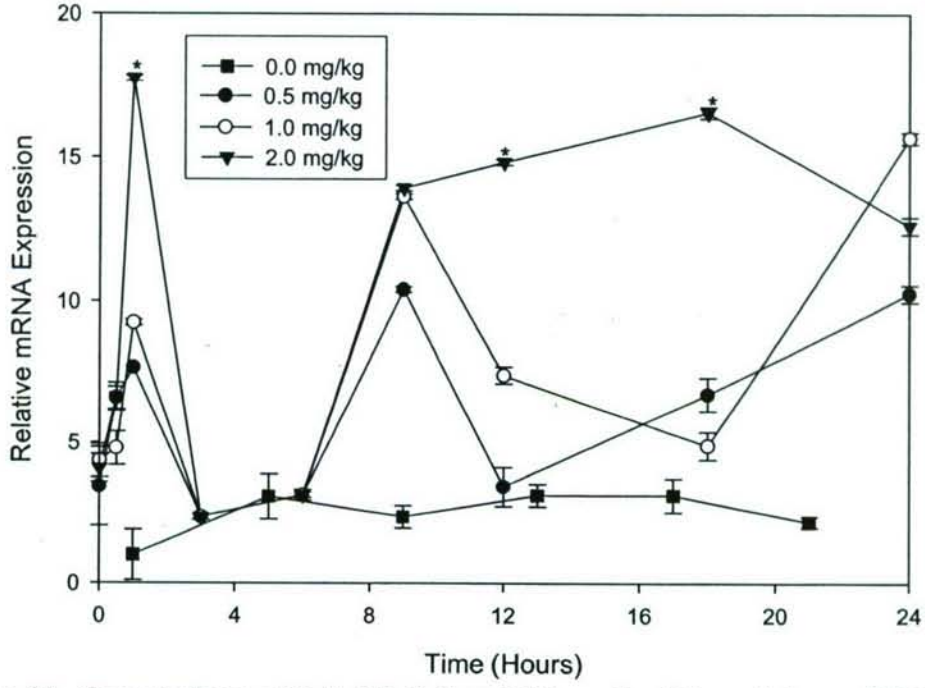

Figure 13. Comparison of 0.0, 0.5, 1.0 and 2.0 mg/kg Cd acetate on MT II mRNA expression

*Indicates significant difference between the means of the 0.5, 1.0 and 2.0 mg/kg doses at a specified time ($p=0.0001$, $p<0.0001$ and $p=0.0109$). Note: Some standard error bars were smaller than the symbol and do not show ($n = 4$).

To investigate the hypothesis that expression of MT I and MT II mRNA are coordinately regulated, the time courses of MT I and MT II mRNA expression were compared at each dose level. At the 0.5 mg/kg dose, MT II shows an initial peak at 1 hour followed by a decline at 3-6 hours (Figure 14). As MT II decreases, MT I increases at 3-6 hours and then decreases to baseline levels at 12 hours. MT II then peaks again at 9 hours. Both MT I and MT II decrease to baseline levels at 12 hours and start to increase again after 12 hours. Note that the levels of MT I expression were significantly higher than MT II throughout the time course. The 1.0 mg/kg dose exhibits a pattern of mRNA expression that was similar to the 0.5 mg/kg dose.

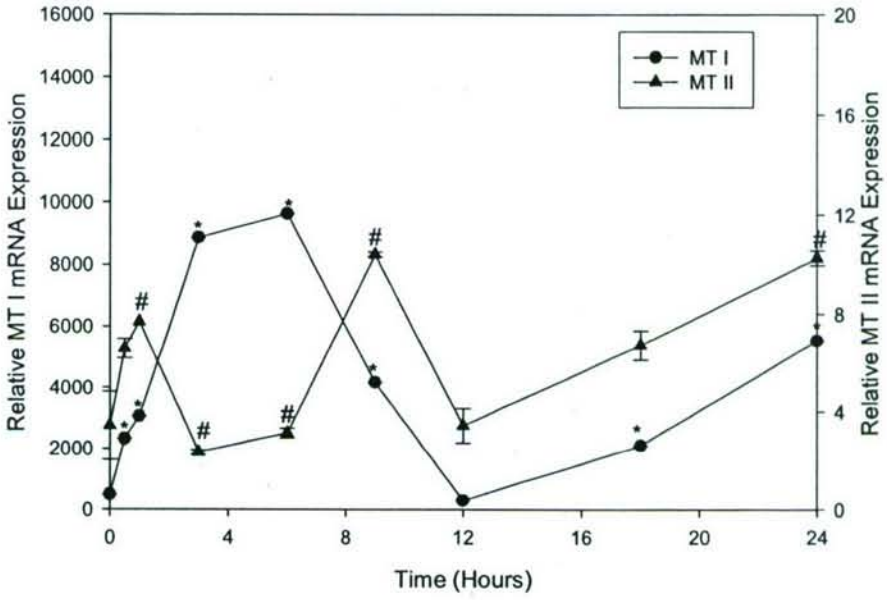

Figure 14. Comparison of MT I and MT II mRNA expression after administration of 0.5 mg/kg Cd acetate

*Indicates significant difference between MT I at various time points as compared to the 0 hour ($p<0.0001$). #Indicates significant difference between MT II at various time points as compared to the 0 hour ($p<0.0001$). Note: Some standard error bars were smaller than the symbol and do not show ($n = 4$).

At a dose level of 1.0 mg/kg Cd acetate, MT II displays a peak at 1 hour followed by a decrease at 3-6 hours (Figure 15). During the decline of MT II mRNA expression, MT I peaks at 6 hours and then decreases to baseline levels by 12 hours, whereas MT II peaks again at 9 hours followed by a decrease to near baseline level at 18 hours. Both MT I and MT II increase again at 24 hours.

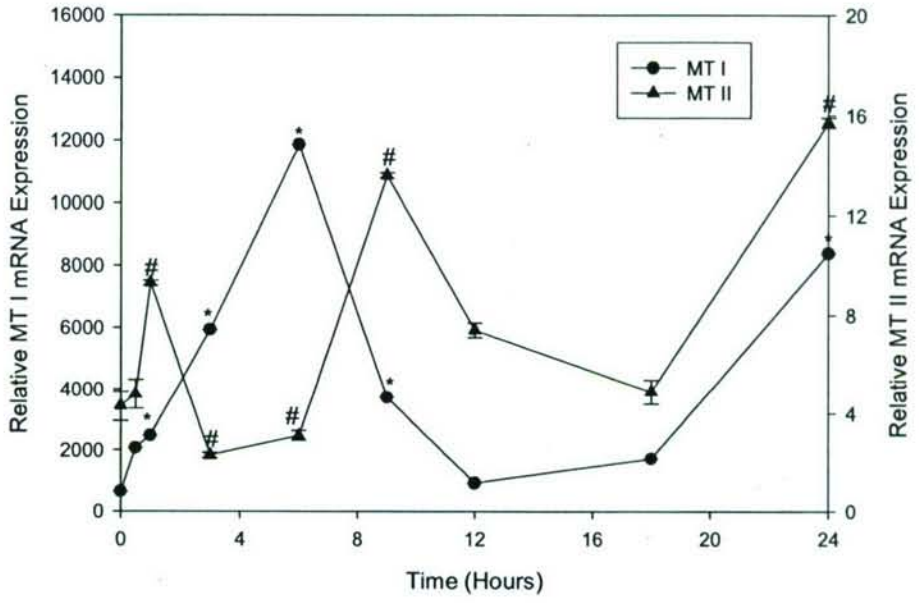

Figure 15. Comparison of MT I and MT II mRNA expression after administration of 1.0 mg/kg Cd acetate

*Indicates significant difference between MT I at various time points as compared to the 0 hour ($p<0.0001$). #Indicates significant difference between MT II at various points as compared to the 0 hour ($p<0.0001$). Note: Some standard error bars were smaller than the symbol and do not show ($n = 4$).

At the highest dose (2.0 mg/kg) both MT I and MT II mRNA expression follow similar patterns to that observed at the 0.5 mg/kg and 1.0 mg/kg doses up to 9 hours (Figure 16). MT II peaks at 1 hour followed by a decrease at 3-6 hours. MT I peaks at 6 hours followed by slight decrease at 9 hours. MT II peaks for a second time at 9 hours. Both MT I and MT II remain elevated from 9-18 hours.

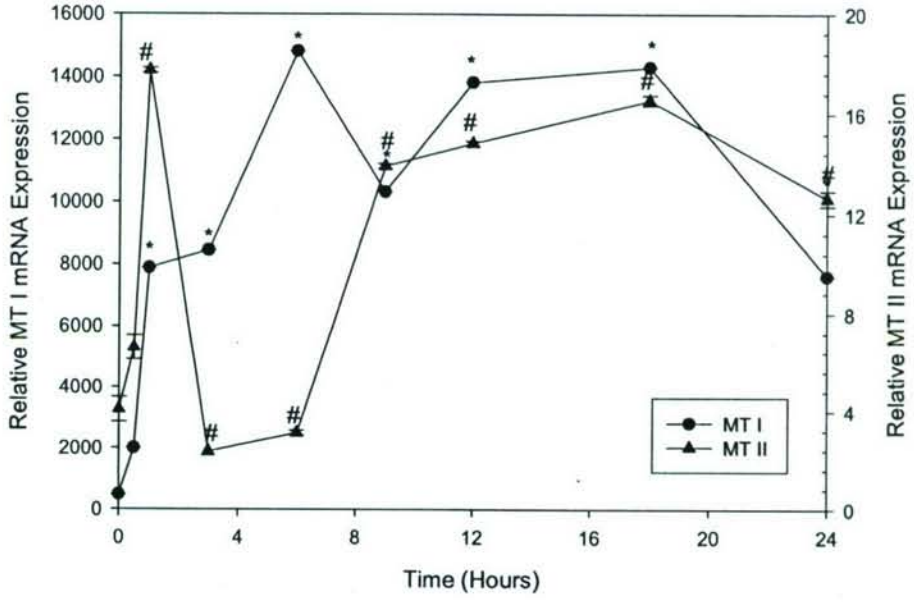

Figure 16. Comparison of MT I and MT II mRNA expression after administration of 2.0 mg/kg Cd acetate

*Indicates significant difference between MT I at various time points as compared to the 0 hour ($p<0.0001$). #Indicates significant difference between MT II at various points as compared to the 0 hour ($p<0.0001$). Note: Standard error bars were smaller than the symbol and do not show ($n = 4$).

By comparing the time courses of both MT I and MT II mRNA expression, the coordination of the regulation of the expression of the two isoforms was evaluated. The data strongly suggest that at early time points up to 12 hours after Cd injection, the expression of the two isoforms was not coordinated. At later time points, after 12 hours, the two isoforms showed similar patterns of expression although the levels of mRNA expression were quite different.

Rat Metallothionein Isoform Regulation *In Vitro*

MTT analysis was used to determine viability of the hepatocytes following treatment. Viability measurements were used to determine the maximum tolerated dose of 10 μM Cd acetate. The MTD was then used to determine the study doses (5.0, 10.0 and 15.0 μM). MTT measurements were also used to calculate the EC_{50} level (i.e., 50% viability decrease as compared to controls) for F344 hepatocytes exposed to Cd acetate. The EC_{50} value was 22.0 μM.

Real Time PCR Validation Study

Before the treated liver samples could be analyzed by Real-Time PCR, a validation experiment was performed to demonstrate that the PCR efficiencies of the target and reference were approximately equal. The absolute value of the slope should be less than 0.1. Proving that the efficiencies of both the target and reference rates of transcription were approximately equal allowed the use of the comparative C_T method for quantitation. To determine if the two efficiencies were equal, varying concentrations of total RNA were analyzed by Real Time PCR for MT I, MT II and GAPDH using the forward and reverse primers.

The average MT I C_T values were calculated along with the ΔC_T value (Table 6). The total RNA was plotted in log ng versus the ΔC_T for MT I (Figure 17). The absolute value of the slope was less than 0.1, which meets the performance criteria.

MT II was analyzed using the same criteria as MT I. The average C_T values for MT II and GAPDH were calculated along with the ΔC_T (Table 7). The total RNA in log ng was plotted versus the ΔC_T and the slope was determined to be -0.0981 (Figure 18). The slope was within the acceptable parameter range.

Table 6. Average C_T Values for MT I and GAPDH in rat hepatocytes cultured *in vitro*

Dilution	Total RNA (ng)	MT I Average C_T	GAPDH Average C_T	Total RNA (log ng)	ΔC_T (MTI -GAPDH)
128	3.91	17.41±0.4	18.78±0.5	0.6	-1.37±0.6
256	1.96	18.31±0.4	19.77±0.5	0.3	-1.46±0.6
512	0.98	19.32±0.4	20.86±0.7	0.0	-1.54±0.8
1024	0.49	20.25±0.4	21.75±0.6	-0.3	-1.50±0.7
2048	0.25	21.28±0.5	22.81±0.8	-0.6	-1.53±0.9
4096	0.13	22.29±0.5	23.84±0.6	-0.9	-1.55±0.8
8192	0.07	23.29±0.5	24.82±0.6	-1.2	-1.53±0.8
16384	0.04	24.28±0.6	25.44±0.6	-1.4	-1.16±0.8
Slope		-0.0384			
Absolute Value		0.0384			

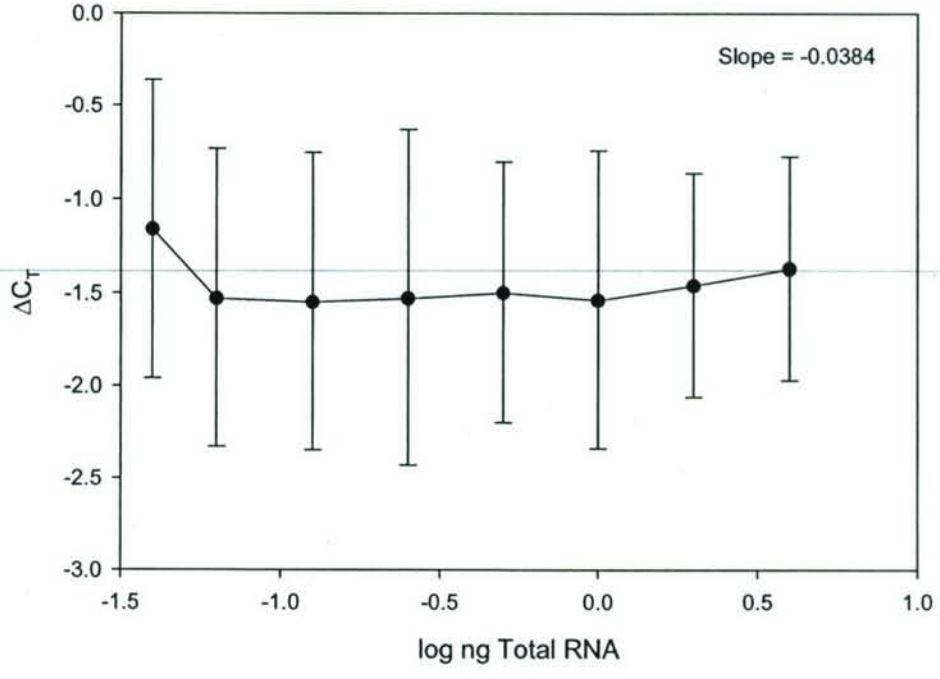

Figure 17. Relative efficiency plot of MT I and GAPDH in rat hepatocytes cultured *in vitro*

Table 7. Average C_T values for MT II and GAPDH in rat hepatocytes cultured *in vitro*

Dilution	Total RNA (ng)	MT I Average C_T	GAPDH Average C_T	Total RNA (log ng)	ΔC_T (MTI -GAPDH)
128	3.91	17.74±0.4	18.78±0.5	0.6	-1.04±0.6
256	1.96	18.77±0.5	19.77±0.5	0.3	-1.00±0.7
512	0.98	19.75±0.5	20.86±0.7	0.0	-1.11±0.9
1024	0.49	20.66±0.5	21.75±0.6	-0.3	-1.09±0.8
2048	0.25	21.62±0.5	22.81±0.8	-0.6	-1.19±0.9
4096	0.13	22.67±0.5	23.84±0.6	-0.9	-1.17±0.8
8192	0.07	23.60±0.6	24.82±0.6	-1.2	-1.22±0.8
16384	0.04	24.42±0.6	25.44±0.6	-1.4	-1.02±0.8
Slope		0.0542			
Absolute Value		0.0542			

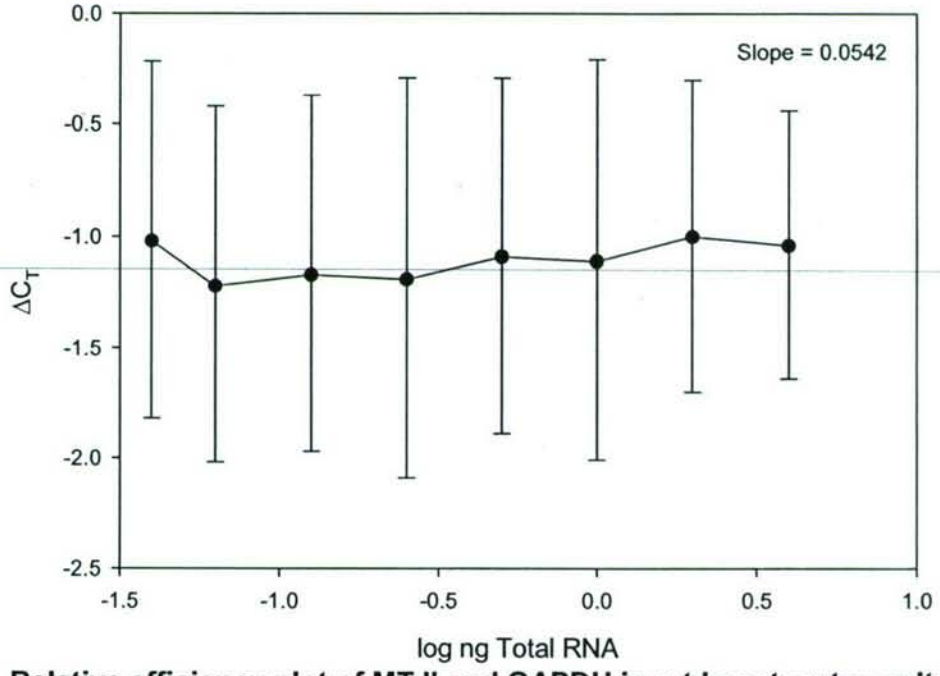

Figure 18. Relative efficiency plot of MT II and GAPDH in rat hepatocytes cultured *in vitro*

Effects of Cd on MT I and MT II mRNA Expression *In Vitro*

The time course of MT I and MT II mRNA expression following rat hepatocyte exposure to 0.0, 5.0, 10.0 or 15.0 μM Cd acetate was determined using Real-Time PCR. The MT I mRNA expression time course data indicate that the MT I mRNA levels peaked at 10 hours for the two

high doses, whereas MT I expression peaked at the beginning of the exposure period for the 5.0 μM dose (Figure 19). The expression levels followed a dose-response pattern. None of the treated cells attained baseline MT I levels before the culmination of the experiment; however, MT I expression declined throughout the study following the sham dosing in the control cells.

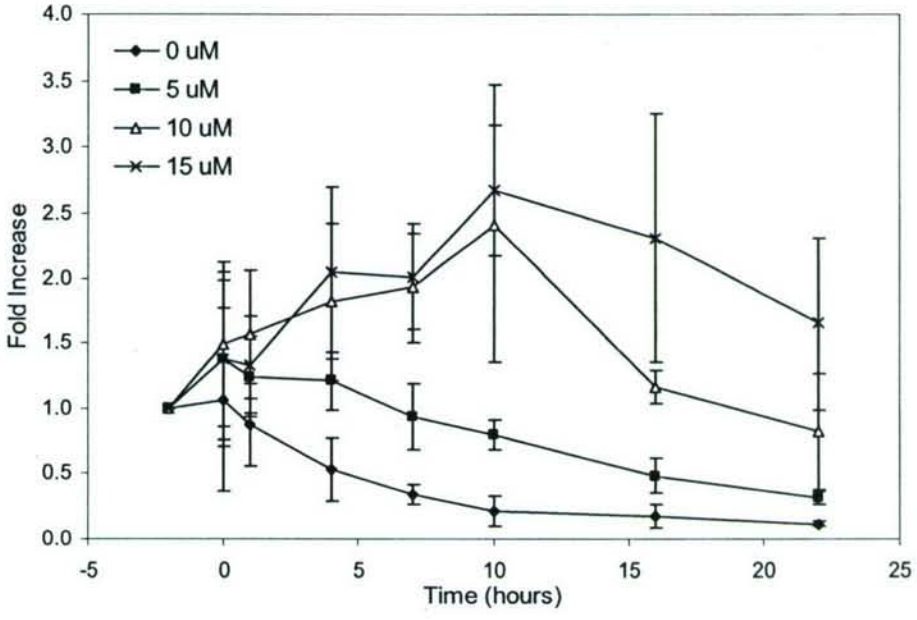

Figure 19. *In vitro* production of MT I in rat liver cells exposed to 0, 5, 10 or 15 μM Cd

The time course data for MT II mRNA expression exhibited a similar pattern than that observed for MT I. MT II mRNA levels peaked at 10 hours for the highest dose, 4 hours for the 10 μM dose and the beginning of the exposure period for the 5.0 μM dose (Figure 20). Like MT I, MT II mRNA expression levels followed a dose-response pattern. Again, none of the treated cells attained baseline MT I levels before the culmination of the experiment. MT II expression declined throughout the study following the sham dosing in the control cells.

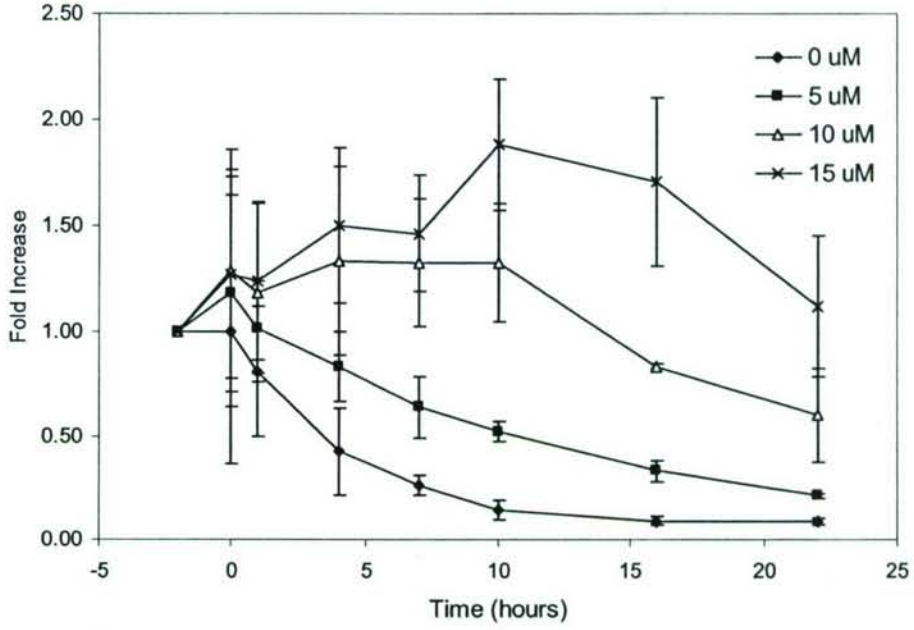

Figure 20. *In vitro* production of MT II in rat liver cells exposed to 0, 5, 10 or 15 μM Cd

DISCUSSION

In Vivo Time Course Kinetics and Liver Effects of Cadmium Exposure

The patterns of Cd concentration over time were determined in serum, liver and kidney in the Fisher 344 rat after *iv* administration at doses of 0, 0.5, 1.0, 2.0 or 3.0 mg/kg. There were some data in the literature for Cd *iv* time-course kinetics in other rat strains (Wistar) (Frazier, 1980), but little to no data for this particular strain of rat. Harstad and Klaassen (2002) published a rat strain comparative study of Cd kinetics after *iv* dosing in the Fisher 344 rat and SD rat, but they did not publish Cd serum time course data and had a very limited number of dose groups, instead choosing to emphasize the difference in response between the strains at only one dose, 2.0 mg/kg, for liver concentration. The maximum liver concentration data at 2.0 mg/kg in Harstad and Klaassen compared very favorably to that in this study, with a peak concentration of ~18 μg/g liver in their study, while the present study observed a similar value of ~20 μg/g liver. A significant difference between the two studies was that, in the present study, the liver concentration increased with time until the final time point. The study by Harstad and Klaassen showed the liver concentration to peak at the first time point (1 hour) and then there was a very minimal drop in concentration over time.

The study by Frazier (1980) presented plasma Cd concentration curves at four doses over an 18 hour time period, but did not evaluate the corollary concentration data for the liver and kidney tissues. Also, the Frazier study only dosed the animals up to ~1.5 mg/kg. The clearance curves in the Frazier study and this study had very similar clearance patterns. A detailed analysis of the data sets from both papers would be required to determine if there are any differences in the kinetics of Cd between the two studies or if there is enough overlapping data

to allow a conclusion about differences of metallothionein and Cd handling between the two different rat strains used in the respective studies.

The concentrations of serum liver enzymes AST and ALT are useful indirect measures of acute liver damage. ALT is a relatively specific indicator of acute liver cell damage as this enzyme predominately appears in liver cells. When liver cell damage occurs, ALT is released from the liver cells into the bloodstream, resulting in abnormally high serum levels that may not return to normal for days or weeks. Measurement of both ALT and AST helps distinguish between heart and liver tissue damage. In this study it was not possible to determine the extent of liver damage in the 3.0 mg/kg dose group using serum ALT and AST levels due to the exceptionally high enzyme concentrations initially determined in the 1/10 normal dilution of the initial sample. There was inadequate sample available to provide the repeated dilutions necessary to bring the serum samples into range of the assay.

Serum ALT values in the 2.0 mg/kg dose group showed a significant response compared to the 0.5 and 1.0 mg/kg dose groups and overall showed the same trend in response as seen in the Harstad and Klaassen (2002) study. The 2.0 mg/kg dose group in the present study did have a more marked response than the two lower dose groups and the data of the Harstad and Klaassen study. Harstad and Klaassen did not sample between 10 and 18 hours post dosing. The added 24 hour sampling point in the present study gave a clearer representation of the time course of ALT response at a higher dose than seen in the previously published study. One significant difference between the present data and that of the Harstad and Klaassen study was the steady high level of both ALT and AST in the present study, while the Harstad and Klaassen study showed a decrease in ALT levels by the 24 hour time point, followed by a continued decrease out to 48 hours, a sampling time point not conducted in the present study. This trend in differences between the two studies suggests there may have been some slight differences in the degree of liver tissue response between these 2.0 mg/kg dose groups, but considering the great inter-laboratory variability that occurs in these types of studies, there was still a significant degree of concurrence of results for these types of end points.

The histopathology results of the liver sections from Cd dosed rats provided visual confirmation of the spectrum of hepatocellular tissue damage seen across the four dose groups, from little to no pathology in the 0.5 mg/kg dose group, to severe hepatocellular toxicity exhibited in the 2.0 and 3.0 mg/kg dose groups. Similar results were reported in the Harstad and Klaassen (2002) study, in which they confirmed that the F344 rat was a more sensitive responder to Cd insult than the SD strain. The same general pattern of liver response was seen in the present study as has been previously reported, that of focal inflammation at the lower dose levels, to congestion and various degrees of focal necrosis at the higher doses. The general patterns of response in this study were well correlated with the time-course of serum enzyme concentration.

Rat Metallothionein Isoform Regulation *In Vivo*

It was the goal of the *in vivo* study to determine if the two isoforms are coordinately regulated. Using Real-Time PCR, it was possible to observe the expression of both MT I and MT II mRNA and evaluate their differences and similarities.

Both MT I and MT II mRNA expression were induced after administration of Cd. It was shown that both time and dose significantly affected the mRNA expression of both the isoforms. Even though both MT I and MT II showed a time and dose effect, their patterns of expression were

very different. MT I showed a biphasic pattern at both the 0.5 mg/kg and 1.0 mg/kg doses. There was an initial peak at 6 hours followed by a return to baseline at 12 hours and a slight increase at 24 hours. This biphasic response has been reported in other studies of MT I mRNA expression. It has been suggested that the second phase is caused by an inflammatory reaction to the toxic effects of the Cd injection (Vascondelos *et al.*, 1996). However the results of this study were inconclusive. The mechanistic basis of the second induction phase of MT I still remains unclear. At the highest dose in this portion of the study, 2.0 mg/kg, the time course of MT I mRNA induction did not exhibit this same biphasic response but instead peaked initially at 6 hours and remained elevated until 24 hours. It can be postulated that at the 2.0 mg/kg dose, a serious inflammatory response had occurred due to the toxicity of Cd and that this could explain why the level of mRNA expression did not return to baseline as it did at the lower doses.

In contrast to MT I, MT II mRNA levels exhibited a triphasic response at both the 0.5 mg/kg and 1.0 mg/kg doses. In the literature, MT II was shown to exhibit a biphasic response similar to MT I but this conclusion is not contradictory as the earliest time point measured was 6 hours (Vascondelos *et al.*, 1996). MT II showed a rapid response at 1 hour followed by a decline to baseline, another peak at 9 hours followed by another decline and finally a slight increase at 24 hours. As was observed with MT I mRNA levels, the 2.0 mg/kg dose did not exhibit the same response as the lower doses; following the second peak at 9 hours, MT II remained elevated until 24 hours. In fact at the 2.0 mg/kg dose, MT II displayed a more biphasic response.

In evaluating the responses of MT I and MT II at each dose level, it was evident that prior to 12 hours both MT I and MT II displayed very different patterns of mRNA expression. The effects were particularly prominent at the two lower doses where toxicity played a minor role. MT II peaked twice at 1 and 9 hours while MT I peaked only once at 6 hours. One possible explanation is that the isoforms exhibit different rates of mRNA synthesis and/or degradation. MT I exhibited a relatively slower rate of synthesis, taking MT I about 6 hours to reach its peak and another 6 hours to return to baseline levels. MT II exhibited a more rapid induction followed by a rapid decline. MT II peaked after 1 hour, returned to baseline at 3 hours followed by another rapid induction at 9 hours and a return to baseline at 12 hours. Thus, MT II mRNA levels appeared to respond more rapidly than MT I. MT II displayed a degradation of t ½ less than 1.5 hours. Although it is often assumed that synthesis and degradation of mRNA are independent, it is possible that whatever factor is responsible for upregulation of the MT II gene may also be responsible for concomitant downregulation of the MT I gene.

It should be noted that MT I mRNA was always present at a much greater quantity than MT II mRNA and MT I mRNA induction was also of a much greater magnitude on an absolute scale. Previous studies have shown that the induction of MT I mRNA was considerably greater than that of MT II (10-fold as opposed to 3-fold) (Vascondelos *et al.*, 1996). One theory to support this is that MT I and MT II proteins may have different affinities for different metals and hence lead to a differential response to these metals at the mRNA level. In humans MT II differs from MT I in amino acid composition and this difference in amino acid composition affects the metal binding affinity of the two isoforms. In humans, MT I is rich in Cd and MT II is rich in Zn (Nordberg and Nordberg, 2000). Other studies have shown a different pattern of MT mRNA expression when comparing Cu and Cd exposure (Vascondelos *et al.*, 1996). Cu induced MT II mRNA more than MT I mRNA and exhibited a monophasic response which remained elevated up to 12 hours. Cd on the other hand induced MT I mRNA more than MT II mRNA exhibiting a biphasic response in which the first phase was over by 12 hours. This latter response is similar to the results obtained in these experiments. These observations support the theory that the isoforms exhibit metal preferences.

The observations found in this *in vivo* study did not support the hypothesis that the two isoforms of MT are coordinately regulated. Instead it supported the idea that the two isoforms play different roles in the detoxification of heavy metals and that different factors may play a role in their mRNA induction. Since MT I and MT II are encoded by different genes, there is some evidence that the expression of some of these genes is under separate control and may serve different biological purposes (Sadhu and Gedamu, 1988; Jahroudi *et al.*, 1990). This may help to explain the differences in both the isoforms mRNA pattern of expression for both isoforms.

Rat Metallothionein Isoform Regulation *In Vitro*

The purpose of the *in vitro* study was to determine if MT mRNA analysis in hepatocytes could be used to predict *in vivo* results. As in the *in vivo* study, both MT I and MT II mRNA expression were induced after administration of Cd acetate *in vitro*. Time and dose significantly affected the mRNA expression of the isoforms. However, Figures 19 and 20 clearly have different overall shapes as compared to Figures 12 and 13. The *in vivo* figures indicate differential regulation of MT I and MT II mRNA, which disproved the hypothesis being tested with the *in vivo* study. The MT I and MT II mRNA expression curves *in vitro* were similar to each other and do not reflect the differences in mRNA regulation shown *in vivo*. The *in vitro* study would indicate that MT I and MT II are coordinately regulated. MT mRNA expression in hepatocytes was not representative of *in vivo* regulation. As this conclusion was apparent, statistical analysis was not carried out.

In vivo, MT I and MT II showed multi-phase time-course patterns. The *in vivo* study did not find coordinate regulation of the MT isoforms at early time points up to 12 hours after Cd injection. However, at later time points after 12 hours, the two isoforms showed similar patterns of expression. *In vitro*, only a single phase was exhibited. This phase most closely resembles the final phase (after 12 hours) of MT mRNA expression *in vivo*. Due to direct hepatocyte dosing *in vitro*, the expression of MT mRNA may have bypassed the initial stages of Cd toxicity due to the inflammatory response noted in other studies (Vascondelos *et al.*, 1996). This inflammatory response could explain why the level of mRNA expression in treated cells did not return to control levels. The *in vitro* responses may actually be predicting the later parts of the *in vivo* curves.

Conversely, the lack of similarity between the *in vivo* and *in vitro* MT mRNA responses may be attributable to the different doses in the target tissues. Serum concentrations in the high dose rats (3.0 mg/kg) *in vivo* did not fall to the *in vitro* 15 μM exposure level until 3 to 6 hours after *iv* dosing. Serum concentrations in the 1.0 and 2.0 mg/kg dose groups didn't fall to even the highest *in vitro* exposure level (15 μM, respectively) until 12 to 18 hours post-dosing. Liver concentrations in the high dose rats were five times higher *in vivo* at the earliest time point (0 hours, immediately after dosing) than the highest exposure level *in vitro*. The concentration difference may explain why the *in vitro* responses more closely mimic the later *in vivo* mRNA expressions, where the serum concentrations are more comparable to the levels used *in vitro*.

However, it is not possible to make a quantitative comparison of Cd levels *in vivo* and *in vitro* without taking into account differences in Cd binding between the studies. It is hypothesized that the level of free cadmium in hepatocytes is the determining factor in predicting toxicity (DelRaso *et al.*, 2003). Measurements of free Cd in serum and within the liver cells from an *in vivo* study would be required for comparison with free Cd concentrations in the exposure media and within hepatocytes from the comparable *in vitro* study. Higher doses *in vitro* may be needed to simulate the Cd dose to liver cells *in vivo*.

CONCLUSIONS

Data generated by this study were collected to provide data for refining and validating a BBK model for Cd in the rat. It was intended that these data be used to make specific comparisons of: (1) average metal concentrations in tissues, plasma, RBC, urine and feces, (2) metallothionein mRNA concentrations in liver, (3) MT concentrations in liver, and (4) the distribution of cadmium and Zn between high molecular weight macromolecules and metallothionein in hepatic cytosol as functions of time and dose. Although some of these data are scattered in the literature, there are no complete studies following an *iv* injection that determine all of these parameters in the same animals over the time course required for these validation studies. Kinetic data produced in this project now need to be used to improve the BBK model for Cd.

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